The complexity of eukaryotic multicellular organisms relies on evolutionary developments that include compartmentalization, alternative splicing, protein domain fusion and post-translational modification. Mammalian gephyrin uniquely exemplifies these processes by combining two enzymatic functions within the biosynthesis of the Moco (molybdenum cofactor) in a multifunctional protein. It also undergoes extensive alternative splicing, especially in neurons, where it also functions as a scaffold protein at inhibitory synapses. Two out of three gephyrin domains homologous to bacterial Moco-synthetic proteins (G and E domains) while being fused by a third gephyrin-specific C domain. In the present paper, we have established the in vitro Moco synthesis using purified components and demonstrated an over 300-fold increase in Moco synthesis for gephyrin compared with the isolated G domain, which synthesizes adenylylated molybdopterin, and E domain, which catalyses the metal insertion at physiological molybdate concentrations in an ATP-dependent manner. We show that the C domain impacts the catalytic efficacy of gephyrin, suggesting an important structural role in product–substrate channelling as depicted by a structural model that is in line with a face-to-face orientation of both active sites. Our functional studies demonstrate the evolutionary advantage of domain fusion in metabolic proteins, which can lead to the development of novel functions in higher eukaryotes.

Key words: alternative splicing, domain fusion, gephyrin, molybdenum cofactor biosynthesis, molybdenum insertion, product–substrate channelling.

INTRODUCTION

Molybdenum forms the active centre in all Mo-enzymes (molybdenum enzymes) [1], catalysing key redox reactions in different metabolic circuits [2]. With the exception of nitrogenase, in all Mo-enzymes, molybdenum is chelated and biologically activated by the Moco (molybdenum cofactor) with molybdenum covalently bound via the dithiolate moiety of a pterin backbone referred to as molybdopterin or MPT (metal-binding pterin) [3]. More than 50 Mo-enzymes have been described so far, but only four are known in mammals [4,5]. Among these, SO (sulfite oxidase) is localized to the mitochondrial intermembrane space [6], as it is crucial for the survival of the organism owing to its function in the detoxification of sulfite, which is mainly generated throughout the catabolism of cysteine residues [7,8]. The other three Mo-enzymes in humans are xanthine dehydrogenase [9], aldehyde oxidase [10] and mARC (mitochondrial amidoxime-reducing component) [11].

In all organisms, Moco is synthesized by a conserved biosynthetic pathway [4] that can be divided into four steps, according to the biosynthetic intermediates cPMP (cyclic pyranopterin monophosphate), MPT and MPT-AMP (adenylylated MPT). Biosynthesis starts with the conversion of GTP to cPMP [12,13] followed by the transfer of two sulfur atoms catalysed by the hetero-tetrameric MPT synthase creating the ene-dithiolate of MPT [14,15]. In eukaryotes, the two last steps of Moco biosynthesis are catalysed by two-domain proteins, with gephyrin [16] and Cnx1 [17] representing two prototypes of this protein family in mammals and plants respectively. They contain two conserved catalytic domains (G and E domains) homologous to the Escherichia coli proteins MogA [18] and MoeA [19].

In vitro studies with the isolated domains of plant Cnx1 have uncovered the principal reaction path of metal insertion into MPT [20]. In the first step, the G domain binds MPT and catalyses the formation of an adenylate at the MPT phosphate followed by the subsequent transfer of the reaction product MPT-AMP to the E domain [20–22]. Finally, molybdate-dependent hydrolysis of MPT-AMP by the E domain results in the formation and release of Moco [20]. However, a verification of these individual steps in a full-length protein is still lacking [23]. In general, domain fusion in Cnx1 and gephyrin is assumed to provide an evolutionary benefit in facilitating the metal insertion reaction in Moco synthesis. A Cnx1-related mechanism of Moco synthesis can be assumed for gephyrin, as it is able to reconstitute Cnx1 function in plants [16,24]. However, gephyrin exhibits significant structural differences to the plant protein Cnx1. Both catalytic G and E domains are oriented in gephyrin in an inverse order and connected by a much larger C domain, which is also subject to extensive alternative splicing [25].

Besides its function in Moco biosynthesis, gephyrin is essential for the clustering of inhibitory glycine and γ-amino butyric acid type A receptors at the postsynaptic membrane in the central nervous system [25]. The gephyrin gene is ubiquitously expressed and undergoes extensive tissue-specific alternative splicing owing to at least ten alternatively spliced exons, as found in rat [26–28], mouse [29] and human [30]. Gephyrin isoforms with or without an additional C1 or C4 cassette within the C domain are the most common variants [25]. We have shown previously that all variants that contain an intact G and E domain are capable of catalysing Moco synthesis in vivo [24] and both domains are also essential for receptor clustering in the brain [31,32].

In the present paper, we demonstrate gephyrin-catalysed molybdenum insertion into de novo synthesized MPT using a fully defined in vitro system. We monitored metal insertion via quantitative reconstitution of human apoSO₄(M) (apo-SO molybdenum) domain with de novo synthesized Moco. In contrast with...
the separated domains (G and E), only full-length gephyrin was able to produce Moco at physiological molybdate concentration in an entirely ATP-dependent manner. Alterations in the catalytic efficiency in different splice variants suggest an important function of the C domain, probably controlling the conformation of both the G and E domains. Consequently, shortening of the C-domain resulted in a length-dependent reduction in activity supporting a structural model in which both active sites face each other for efficient product–substrate channelling.

**EXPERIMENTAL**

**Molecular biology**

Details of the plasmids used in the present study are listed in (Supplementary Table S1 at http://www.biochemj.org/bj/450/bj4500149add.htm). SO full-length cDNA (GenBank® accession number BC065193) was purchased from Imagenes and recombinant human SO was generated by cloning the coding sequence for the dimerization and Moco domains (nucleotides 499–1638) into the SalI/HindIII restriction sites of pQE80L (QIAGEN). Gephyrin sequence (GenBank® accession number NM_022865) was used to generate three Sf9 insect cell expression constructs: gephyrin P1, gephyrin C3 and gephyrin C4; and three E. coli expression constructs: gephyrin C1; geph-G (gephyrin G) domain and geph-E (gephyrin E) domain. All E. coli gephyrin constructs were cloned into pQIE80L expression plasmid using KpnI and HindIII for full-length gephyrin and SalI and HindIII restriction sites. Sf9 insect cell constructs have been described recently [33].

**Protein expression and purification**

cPMP and the E. coli MPT synthase subunits MoaE and MoaD were purified as described previously [34,35]. Human apoSO₃₅ was expressed in the E. coli moaC strain RRK5245 [36]. Expression was induced with 0.1 mM isopropyl β-D-thiogalactosidase at Dₗ₀ = 0.5 and continued for 15 h at 30 °C. His-tagged proteins were purified by Ni-NTA (Ni²⁺-nitrilotriacetic acid) affinity according to the manufacturer’s (QIAGEN) instructions. All geph-G and -E domain constructs were expressed in E. coli BL21 (DE3) and purified using Ni-NTA and anion-exchange chromatography. Gephypyrin splice variants were expressed as described in [33]. All purified proteins were exchanged in the same buffer [20 mM Tris/HCl (pH 8.0) and 50 mM NaCl] using PD-10 desalting columns (GE Healthcare) and stored at −80 °C.

**Determination of MPT, MPT-AMP and Moco content**

MPT/Moco and MPT-AMP were first oxidized to their stable oxidation products FormA-dephospho and FormA-AMP respectively and further quantified using HPLC reverse-phase chromatography as described previously in [20].

**MPT in vitro transfer to gephyrin C₄**

MPT in vitro synthesis was performed in the presence of gephyrin C₄, which was expressed in E. coli. A mixture of MoaD (800 nmol), MoaE (30 nmol), cPMP (300 nmol) and gephyrin C₄ (100 nmol) was prepared in 100 mM Hepes buffer (pH 7.5) and incubated at room temperature (22 °C). After 30 min, the reaction mixture was separated by ultra-filtration under anaerobic conditions using Amicon Ultra 100-kDa centrifuge filters (Millipore), and an aliquot (1 %; 5 μl) was analysed by size-exclusion chromatography using an Acquity UPLC BEH200 1.7-μm column (Waters), equilibrated with 100 mM KH₂PO₄ buffer (pH 6.8). The gephyrin C₄ peak was identified by comparing its retention time with that of an injected MPT-free gephyrin C₄ (40 pmol).

**MPT synthesis and adenylation reactions**

A mixture of thio-carboxylated MoaD and MoaE [35] was incubated in the absence or presence of either gephyrin C₄ (200 pmol) or geph G domain (200 pmol) and MPT synthesis was started by the addition of 500 pmol of purified cPMP [34]. After 1 h, adenylation of MPT was started by the addition of 10 mM Mg²⁺ and 2 mM ATP and then incubated for 1 h. The reactions of MPT synthesis and adenylation were stopped at different time intervals and quantified by HPLC FormA analysis.

**Moco in vitro reconstitution assay**

All reactions were performed at room temperature in 100 mM Hepes buffer (pH 7.5). First, a mixture of MoaD (3 nmol), MoaE (0.1 nmol), gephyrin or gephyrin domains (1 nmol) and molybdate (various amounts) was prepared. If ATP influence was investigated, 10 mM MgCl₂ and (if not otherwise stated) 2 mM ATP were added and the reaction was started by the addition of cPMP (1 nmol), which was purified as described previously in [34]. Following an incubation time t₁ of 20 min, the second step was initiated by the addition of apoSO₃⁺ (1 nmol) followed by an incubation time t₂ of 20 min. Reconstituted SO₃⁺ activity was determined using the potassium ferricyanide [K₃[Fe(CN)₆]] assay. Potassium ferricyanide (20 μM) was added and the reaction was started by the addition of sodium sulfite (300 μM). SO activity was quantified by monitoring the reduction of potassium ferricyanide at 420 nm (absorption coefficient of potassium ferricyanide at 420 nm ε = 19200 M⁻¹ cm⁻¹) using a 96-well-plate reader (BioTeK). The activity of the SO₃⁺ protein was determined in μM/min and correlated with the amount of bound Moco based on the known saturation of in vivo assembled SO₃⁺ using FormA HPLC analysis.

**Structure modelling of full-length gephyrin**

Given the fact that gephyrin, when expressed in E. coli, forms stable trimers, we assumed a symmetrical trimer mediated by the known tight interaction of the G domains [37,38]. Consequently, and in contrast with the known crystal structure [37,38], all three E domains should make contacts with a G-domain monomer. Using the structural similarities of the G domain to the subdomain 3 of the E domain, we superimposed an E domain with all three G domains of the trimer (PDB 1JLJ) [37] with an E-domain monomer (PDB 2FU3) [39] using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4). Following superimposing of each E-domain monomer, the other monomer as oriented in the dimer was created for each of the overlaid E domains. The positions of these dimer pairs were assumed to mimic a possible orientation of the E domain relative to the G domains and were rendered using PyMOL.
with approximately 33% saturation was demonstrated by FormA analysis (results not shown).

After showing that MPT can be successfully transferred to gephyrin C₄ in vitro, we performed analytical MPT synthesis in the presence and absence of geph-G or gephyrin C₄ and monitored the formation of MPT-AMP over time after ATP addition (Figure 2). In the presence of a MoaD, MoaE and cPMP mixture, MPT synthesis was very low in the protein-free control (5 pmol, Figure 2A) and only the presence of either geph-G (Figure 2B) or gephyrin C₄ (Figure 2C) enhanced the MPT synthesis rate by 6-fold. This finding demonstrates the ability of gephyrin C₄ to bind MPT via its G domain and also shows that MPT needs to be liberated from the MPT synthase in order to initiate another cycle of sulfur transfer to cPMP. The formation of MPT-AMP was detected only in the presence of geph-G and gephyrin C₄ when Mg²⁺ (10 mM) and ATP (2 mM) were added to the reaction mixture (Figures 2B and 2C). Gephyrin C₄ showed a higher MPT-AMP formation compared with geph-G and MPT-AMP formation correlated with a decrease in remaining MPT (Figures 2B and 2C). These results demonstrate that gephyrin C₄ catalyses the adenylylation of MPT via its G domain similar to its homologous plant protein Cnx1 in a Mg²⁺ and ATP-dependent reaction.

**In vitro synthesis of Moco using purified components**

In order to elucidate the function of gephyrin domains in Moco biosynthesis, we developed a fully defined in vitro system using purified components (Figure 3). The set-up covered three out of four reaction steps in Moco biosynthesis: MPT synthesis, MPT adenylylation and metal insertion. MPT was synthesized de novo from purified cPMP [34], using the E. coli proteins MoaE and thiocarboxylated MoaD [35]. The following two steps of Moco biosynthesis were carried out using the separated geph-G and geph-E domains expressed in E. coli (Figure 3). To monitor the formation of Moco, human SO₃mo was expressed in the E. coli moaC mutant strain RK5245 [36], which is defective in the first step of Moco biosynthesis. Consequently, purified apoSO₃mo was free of Moco and therefore not active (Supplementary Figure S1 at http://www.biochemj.org/bj/450/bj4500149add.htm). The formation of Moco was monitored via the activity of reconstituted SO₃mo enzyme and correlated with the amount of bound Moco.

**RESULTS**

**Gephyrin C₄ binds MPT and catalyses MPT adenylylation via its G domain**

Using the isolated Cnx1 domains, we have demonstrated previously that Moco synthesis involves the formation of MPT-AMP by the G domain as an intermediate before molybdenum insertion by the E domain. Furthermore, MPT-AMP formation was Mg²⁺ and ATP-dependent [40]. To investigate the function of full-length gephyrin in a fully defined in vitro system, we used gephyrin C₄, a variant that is highly expressed in liver, kidney as well as neuronal tissue [27] and has been biochemically characterized in previous studies [24,38]. MPT synthesis was performed de novo from purified cPMP [34] using the E. coli proteins MoaE and thiocarboxylated MoaD, which spontaneously assemble into the hetero-tetrameric MPT synthase [35].

First, we performed MPT in vitro synthesis in the presence of gephyrin C₄ and analysed the MPT-loaded gephyrin C₄ complex in comparison with MPT-free gephyrin C₄ by size-exclusion chromatography. Analysis of MPT-free gephyrin C₄ showed a major peak at a molecular mass of 290 kDa corresponding to a gephyrin trimer (Figure 1A). Following in vitro MPT synthesis and co-incubation with gephyrin C₄, a similar elution time of gephyrin C₄ with additional peaks derived from MPT synthase proteins was observed (Figure 1B). Interestingly, the absorption spectrum of gephyrin C₄, derived from the MPT in vitro assay, showed a clear additional absorption maximum at 400 nm, which was absent in the sample of purified gephyrin C₄ (Figures 1C and 1D) and is reminiscent to the absorption spectrum of MPT-loaded Cnx1G protein [22]. Successful MPT transfer to gephyrin C₄...
The second reaction was started by the addition of apoSO\textsubscript{MO} and times \(t\) (Figure 3, step 3). As a starting point, two successive incubation monitoring sulfite-dependent reduction of potassium ferricyanide synthesis, step 2 starts with apoSO\textsubscript{MO} addition. Activity of the reconstituted SO\textsubscript{MO} is measured using the potassium ferricyanide assay in step 3.

Moco insertion into MPT catalysed by gephyrin G and E domains respectively. Following Moco synthesis, step 2 consists of adenylylation and molybdenum. Both conditions showed Moco formation with the highest activity of cPMP with synthesized Moco for binding to the cofactor-binding site of apoSO\textsubscript{MO}. SO\textsubscript{MO} activity started to reach saturation with approximately equimolar amounts produced Moco. Therefore we conclude that our experimental set-up represents truly quantitative \textit{in vitro} synthesis of Moco.

Next we investigated the molybdate-dependent reconstitution of apoSO\textsubscript{MO} in the absence (non-enzymatic) and presence of equimolar amounts (1 nmol) of geph-G and geph-E (Figure 4B). Enhanced Moco-synthesizing activity of full-length gephyrin provides evidence for product–substrate channelling

To date, we have shown that gephyrin C\textsubscript{4} binds MPT via its G domain and catalyses an adenyl transfer to the MPT phosphate, a process that requires Mg\textsubscript{2+}ATP. Furthermore, catalytic Moco synthesis was observed when using the isolated gephyrin domains in the presence of 50 \(\mu\)M molybdate. However, the physiological molybdate concentration is below 1 \(\mu\)M [42] and it is therefore not surprising that we have observed a significant activity in the absence of gephyrin domains owing to chemical ligation, which bypassed the ATP-dependent enzymatic synthesis of Moco. Therefore we reduced the molybdate concentration to 5 \(\mu\)M in the present study, and investigated the ATP-dependent molybdenum insertion activity of gephyrin C\textsubscript{4} (as a model for full-length gephyrin) in comparison with the isolated gephyrin domains geph-G and geph-E (Figure 5).

At 5 \(\mu\)M molybdate, the non-enzymatic activity was ATP-independent and remained very low at all conditions (1–2 pmol Moco, Figure 5B). The addition of geph-G and geph-E domains together also resulted in a very low activity (8–16 pmol Moco compared with \(~500\) pmol Moco in the presence of 50 \(\mu\)M molybdate, Figure 4B and Figure 5B). In contrast, reconstitution with gephyrin C\textsubscript{4} revealed a strong ATP dependence of the reaction and reached an up to 344-fold increase in Moco formation (260 pm Moco) at 10 mM ATP compared with geph-G and geph-E (Figure 5B). In conclusion, the strongly increased ATP-dependent activity of gephyrin C\textsubscript{4} suggests effective product–substrate channelling between both active sites within the holoprotein.
Moco formation was measured at 5 μM molybdate as a function of ATP in the presence of gephyrin C4, gephyrin-G and gephyrin-E or different gephyrin truncated variants. (A) Schematic representation of the gephyrin constructs used in the present study. The first and last residues of the G and E domain are shown in holo-gephyrin. The last and first residues in the truncated chimaeras depict the size of C-domain deletion variants geph-C0, geph-C22 and geph-C87. (B) Moco synthesis was measured in the absence of gephyrin, in the presence of gephyrin domains and in the presence of full-length gephyrin C4. The activities of the truncated gephyrin variants were measured under the same conditions as for full-length gephyrin C4. All gephyrin domain constructs, full-length and truncated variants were expressed in E. coli and 1 nmol of protein was used in each experiment. All values represent average readings of three independent experiments, and error bars indicate the respective S.D.

Alternative splicing of the C domain alters Moco synthesis

Given our finding that full-length gephyrin C4 is approximately two orders of magnitude more active at low molybdate concentrations than the individual domains and that this activity is strongly dependent on ATP, we next investigated three different full-length splice variants: gephyrin P1 (the originally described variant without any additional cassettes in the C domain [16]), gephyrin C3 and gephyrin C4. In contrast with the previous experiments, splice variants were now expressed in Sf9 insect cells, because in E. coli insufficient quantities of gephyrin P1 and C4 were expressed. Expression in Sf9 insect cells resulted in approximately equal levels of all three purified gephyrin splice variants [33].

First, we investigated the ATP dependence of molybdenum insertion and found for all three variants an ATP-dependent increase in activity as seen for E. coli gephyrin C4. Maximal activity was reached with 15 mM ATP. Interestingly, gephyrin P1 and gephyrin C3 were more active at low ATP concentrations than gephyrin C4, and gephyrin P1 showed higher activity in the absence of ATP (Figures 6A–6C). Recently, we showed that Sf9 insect cell-derived gephyrin is phosphorylated at different residues within the C domain. Given the fact that ATP-dependent activities of E. coli- and Sf9-insect-cell-expressed gephyrin C4 were similar in the present study, we conclude that phosphorylation of the C domain of gephyrin does not influence its Moco-synthesizing activity.

Next, we performed molybdate titrations in the presence and absence of 15 mM ATP and found molybdate-dependent Moco synthesis for all variants (Figures 6D–6F). Noteworthy, gephyrin P1 and gephyrin C4 also showed an increase in activity in the absence of ATP but with a much lower rate, whereas with gephyrin C3, no activity was found in the absence of ATP at the used molybdate concentration range. However, gephyrin C3 was the variant with the most efficient response to molybdate. Fitting the ATP-dependent molybdate titrations using the Michaelis–Menten equation resulted in a $K_m$ for molybdate

of holo-gephyrin in comparison with the individual domains, we next asked whether the proposed product–substrate channelling in gephyrin is dependent on the size of the C domain. One could assume that both catalytic domains need to face each other, which in turn requires defined conformation, probably controlled by the C domain. Therefore three chimaeric gephyrin variants with different C domain lengths were created (Figure 5A): gephy-C0, gephy-C22 and gephy-C87. The truncated gephyrin variants were expressed in E. coli and purified in the same way as gephyrin C4. SDS/PAGE analysis of the purified gephyrin truncated variants showed a similar purity profile compared with gephyrin C4, demonstrating that the C domain deletion did not alter the stability of the resulting gephyrin chimaeras (Supplementary Figure S2 at http://www.biochemj.org/bj/450/bj4500149add.htm).

We investigated ATP-dependent Moco synthesis of all three truncated gephyrin variants (Figure 5C). Interestingly, there was a C-domain length-dependent reduction in Moco activity detectable. The deletion of the entire C domain (gephy-C0, Figure 5C) resulted in a very low activity compared with gephyrin C4 and was ATP-independent (Figures 5C and 5B). In contrast, partial deletion of the C domain (gephy-C22 and gephy-C87) resulted in a partial reduction of ATP-dependent activity reaching a maximum of 65 and 75 pmol Moco for geph-C22 and geph-C87 respectively (Figure 5C). Note that gephyrin C4 activity reached saturation at an ATP concentration of 10 mM, whereas the activities of geph-C22 and geph-C87 continued to increase at higher ATP concentrations, demonstrating a loss in the ATP-dependent molybdenum insertion reaction catalysed by both domains (Figures 5C and 5B).
of 4.5 μM ± 1.5 μM for gephyrin P1, 0.8 μM ± 0.3 μM for gephyrin C1 and 0.4 μM ± 0.1 μM for gephyrin C2 (Figures 6D–6F). In contrast, the ATP-independent molybdate titration data could not be fitted using the Michaelis–Menten equation as they followed a linear increase in activity.

Crystal structure modelling supports product–substrate channelling in gephyrin

Although the crystal structure of full-length gephyrin is still lacking, we have used the available co-ordinates of both catalytic domains [37,39] to construct a model of full-length gephyrin (Figure 7, for details see the Experimental section), which is based on E domains dimerizing with each subunit of a G-domain trimer. One subunit of an E-domain dimer was superimposed with the G domain using the structural homology of geph-E subdomain 3 [39]. In the resulting trimer (mediated by G domains), residues important for gephyrin’s neuronal function (i.e. glycine receptor and collybistin binding) are surface exposed on one side of the molecule thus enabling the receptor clustering function at the postsynaptic membrane to occur. Furthermore, there is a 71 Å (1 Å = 0.1 nm) distance between the C- and N-termini of the G and E domain respectively, which could explain the requirement for an extended C domain (Figure 7A, dotted line) to bridge this large gap and would be consistent with our observed loss in activity when the C domain is truncated. The C domain is presumably surface exposed as supported by the known high sensitivity of gephyrin towards proteolytic degradation [25]. In contrast, in the inverted orientation, the N- and C-termini are only 17 Å apart, which is consistent with the very short linker connecting the E and G domain in plant Cnx1 [43].

To visualize the active site of each G domain, MPT-AMP is highlighted as determined by X-ray crystallography in the homologous Cnx1 G domain [22]. In addition, given the known transfer and binding of MPT-AMP in the E domain, MPT-AMP was modelled into the proposed active site of the E domains (Figure 7B, yellow subunit), which is surrounded by functionally important residues [44]. Crystal structures of gephyrin E domain [39] and E. coli MoeA [44] disclosed that subdomain 1 is highly mobile and hosts additional active-site residues [44]. In our model, those residues face the G-domain-bound MPT-AMP in...
a conformation that we call a ‘closed’ state (Figure 7C, orange and yellow subunits). For MPT-AMP transfer to the E domain, subdomain 1 needs to change its conformation, which could be easily accomplished by a ‘swing out’ motion supported by the hinge, connecting subdomain 1 and 2 (Figure 7C, red subunit). As a result, an ‘open’ conformation would form and the inner wall of subdomain 1 might form a channel that could guide MPT-AMP into the second E domain active site, where metal insertion takes place. In conclusion, the resulting model of gephyrin is consistent with both the functional requirements of gephyrin in neuroreceptor clustering as well as its observed high catalytic efficacy compared with the isolated domains.

DISCUSSION

Whereas in E. coli two proteins, MogA [45] and MoeA [46], are required for molybdenum insertion into MPT, in eukaryotes those activities are fused in two-domain proteins such as Cnx1 [17] and gephyrin [16]. Using the isolated catalytic domains of Cnx1, we have uncovered the reaction sequence of molybdenum insertion into MPT, which consists of MPT-AMP synthesis by the G domain [22,40] and molybdate-dependent hydrolysis of MPT-AMP and Moco formation by the E domain [20]. Given the inverse orientations of G and E domains in gephyrin and the presence of an additional C domain (160 residues), two important questions arose: first, what is the functional benefit of having both domains fused in a multifunctional protein and to what extent does this facilitate Moco synthesis; secondly, what is the contribution of the C domain in this process, and in particular, do alternatively spliced variants of the C domain impact the function of gephyrin in Moco biosynthesis?

Nichols and Rajagopalan [47] reported in vitro Moco synthesis using a 10-fold excess of the bacterial MoeA protein over MogA. In the present study, we also observed increased activity with excess geph-E (5-fold) over geph-G, in an ATP-dependent reaction (Supplementary Figure S3 at http://www.biochemj.org/bj/450/bj4500149add.htm). This confirms MPT adenyllylation and/or MPT-AMP transfer as rate-limiting step(s) in the molybdenum ligation reaction, as we have previously shown using Cnx1 G and E domains [20,40]. Furthermore, we observed significant non-enzymatic reconstitution with the same molybdate concentrations (50 μM) as used by Nichols and Rajagopalan [47]. This raised the question of whether these conditions, which are clearly not physiological, are appropriate to study molybdenum insertion and to draw any conclusion on the impact of ATP on this reaction. Consequently, Nichols and Rajagopalan [47] found only a 2-fold increase in activity in the presence of ATP.

When we reduced the molybdate concentration by 10-fold, non-enzymatic molybdate ligation to MPT was largely eliminated. In addition, the activity of the separated gephyrin domains was much lower, whereas the use of full-length gephyrin C4 demonstrated the importance of the ATP-dependent activation of MPT by the G domain. In fact, gephyrin C4-catalysed molybdenum ligation was over 300-fold more efficient as the activity of isolated G and E domains.

Previous adenylylation studies with Cnx1G and geph-G revealed an optimal ATP concentration of 0.5–1 mM [24,40]. Higher concentrations of up to 10 mM ATP resulted in an inhibition of MPT adenylylation, which was due to a stronger back-reaction with increasing amounts of pyrophosphate derived from spontaneous ATP hydrolysis [40]. Also, in the present study, we observed such an effect on Moco synthesis, either with the G and E domains as well as full-length gephyrin. However, for the individual domains, inhibition started at lower ATP concentrations (8 mM) than for gephyrin C4 (20 mM). Given the fact that the experimental set-up used in the present study monitors the entire reaction from MPT to Moco, the overall reaction equilibrium is shifted towards the formation of Moco, resulting in a directed product–substrate (MPT-AMP) flow, which in turn was found to be most effective in holo-gephyrin. As a result, traces amounts of pyrophosphate have a much lower impact on the overall reaction than on the half-reaction catalysed by the G domains.

The fact that full-length gephyrin were less sensitive to high ATP concentration and most active at low molybdate concentrations suggest an effective transfer of MPT-AMP from the G to the E domain, a process that is known as product–substrate channelling in multidomain and multisubunit enzymes. Such a product–substrate channelling in full-length gephyrin would also reduce the cytosolic exposure of MPT-AMP, which is highly sensitive to oxidation [22].

Next, we questioned why full-length gephyrin is more active than the individual domains at low molybdate concentrations? First, a non-enzymatic molybdate chelation to MPT with a $K_d$ of 0.8 mM for molybdate was recorded, which is much lower than the previously reported $K_d$ of 20 mM [48]. Since chemical ligation of molybdate becomes favourable at high molybdate concentrations, ATP-dependent molybdenum insertion will be bypassed and/or inhibited. Secondly, at 2 mM ATP, an equilibrium mixture of G and E domains was able to catalyse ATP-dependent molybdate insertion into MPT with a $K_{	ext{act}}$ of 91 μM, which is one order of magnitude lower than the $K_d$ for chemical ligation. However, with intracellular molybdate concentrations below 1 μM [49], the lack of Moco synthesis at low molybdate concentrations (5 μM) demonstrated the strict requirement of ATP for Moco synthesis under physiological conditions. Thirdly, only the use of full-length gephyrin C4 resulted in approximately two orders of magnitude more efficient molybdenum insertion reaction than for the separated domains, with a $K_{	ext{act}}$ for molybdate as low as 0.4 μM. We conclude that full-length gephyrin provides the native conformation of both the G and E domain for efficient product–substrate channelling, thus disclosing the driving force for the fusion of both functions into a multidomain protein, a process that happened at least twice during evolution [44].

To evaluate the influence of the central domain in the arrangement of the catalytic domains, we created gephyrin variants with different C domain lengths and demonstrated that a full C domain is strictly required for gephyrin function. Consequently, spatial proximity of geph-G and geph-E is not sufficient, given that the geph-C0 variant completely lost its activity in Moco synthesis. The fact that we could tune the activity with different C domain lengths supports our hypothesis of product–substrate channelling in the full-length protein. In line with this finding we observed splice-specific alterations in Moco synthesis.

Whereas splice variants gephyrin P$_1$ and C$_1$ also exhibited some ATP-independent activity, gephyrin C4 was strictly ATP-dependent with the lowest $K_{	ext{act}}$ of 0.4 μM for molybdate. Given the recently determined $K_{	ext{act}}$ for molybdate transport (0.5 μM) [50], we conclude that the gephyrin C4 variant effectively operates at intracellular molybdate concentrations. The fact that gephyrin C4 shows no ATP-independent activity suggests a strong binding of MPT to gephyrin C4 thus blocking non-enzymatic chemical ligation of molybdate. As all three variants only differ in their C-domain composition, these data support a critical role of the C domain in controlling the arrangement of the catalytic domains within full-length gephyrin. We created a structural model for holo-gephyrin, which supports functional properties in Moco biosynthesis as well as neuroreceptor clustering. The model provides a face-to-face orientation of both catalytic sites (G and
E domains), supporting the release of the first reaction product (MPT-AMP) into the site of molybdenum insertion.

In summary, we have reported an in vitro system for gephyrin-mediated synthesis of Moco, a reaction that is strictly ATP-dependent under physiological molybdate concentrations. Alternative splicing or artificial modification of the C domain alters the catalytic properties of full-length gephyrin, thus supporting its crucial function in the orientation of both catalytic domains within the full-length protein. To our knowledge, this is the first experimental evidence demonstrating the functional advantage of domain fusion in eukaryotes in comparison with the separately expressed proteins in prokaryotes. Therefore, gephyrin uniquely exemplifies the evolution of novel protein functions (i.e. receptor clustering compared with basic metabolism) within protein domains being acquired to bridge the two catalytic modules in multidomain proteins.

AUTHOR CONTRIBUTION
Abdel Belaidi designed and performed the experiments, analysed the results and wrote the paper; Guenter Schwarz designed the study, analysed the results and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

Metal insertion into the molybdenum cofactor: product–substrate channelling demonstrates the functional origin of domain fusion in gephyrin

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Figure S1 Sulfite oxidase activity of holo and apoSO_MO

(A) The activity of increasing amounts of holo and apoSO_MO was measured using the FeCN assay. The amount of protein was converted to Moco content that was determined by HPLC FormA analysis. The final figure represents activity of holo and apoSO_MO as a function of Moco saturation. Linear regression for the activity of holoSO_MO is highlighted in the figure and the determined slope was used to determine the correlation between SO activity and Moco content. Triplicate samples were used in each experiment and error bars indicate S.D. nd, not detected.

Figure S2 SDS/PAGE analysis of truncated gephyrin variants

Gephyrin C4 and three truncated gephyrin variants geph-C0, geph-C22 and geph-C87 were analysed on a 10% polyacrylamide gel. All proteins were expressed in E. coli and purifications were performed the same day for all proteins. A total of 10 μg of protein was loaded in each lane and a protein marker was used to identify the molecular mass (kDa) of the protein bands.

Figure S3 ATP-dependent activity of gephyrin G and E domains

Moco formation was measured at 50 μM molybdate in the presence and absence of 2 mM ATP. Geph-G and geph-E were individually expressed in E. coli. Moco formation was measured in the presence of 50 μM molybdate, 1 nmol geph-E, in the presence and absence of 2 mM ATP and increasing amounts of geph-G. Triplicate samples were used in each experiment and error bars indicate the respective S.D.

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## Table S1 Plasmids

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