Asymmetric dimethylation of arginine residues is a common posttranslational modification of proteins carried out by type I protein arginine methyltransferases, including PRMT1 and -3. We report that the consecutive transfer of two methyl groups to a single arginine side chain by PRMT1 and -3 occurs in a distributive manner, i.e. with intermittent release of the monomethylated intermediate. The oligomeric state of PRMTs together with the clustering of methylated arginine residues in most proteins carrying this type of modification suggests that multiple methyl transfers to a single polypeptide chain might proceed in a processive manner by cooperation of multiple active sites. However, three different types of experiments provide evidence that the reaction is distributive even with substrates containing multiple methyl-accepting arginines, including one with 13 such residues. PRMT1 also does not prefer substrates already containing one or more singly or doubly methylated arginine residues. Even though the reaction is distributive, the efficiency of methylation of one particular protein strongly depends on the number of methyl-accepting arginine residues it contains.

Dimethylation of arginine residues is a common posttranslational modification of proteins. The main function of arginine methylation seems to be the modulation of protein-protein interactions (1–3). As one prominent example, the asymmetric dimethylation of arginine residues is one of several modifications contributing to the “histone code” (4–8). Dimethylation of arginine proceeds via the intermediate $\omega-N^G$-monomethylarginine and results in either symmetric $\omega-N^G,N^G$-dimethylarginine or asymmetric $\omega-N^G,N^G$-dimethylarginine. The reaction is catalyzed by protein arginine methyltransferases (PRMTs)". PRMTs are classified into type I and type II enzymes according to their end products (2, 3, 9, 10). Both classes catalyze the formation of monomethylated arginine. The intermediate is converted to symmetric dimethylarginine by type II PRMTs. Mammalian members of this class are PRMTs -7, and -9 (2, 10). The spliceosomal Sm proteins are prominent substrates for PRMT5 (11–14). In contrast, type I PRMTs convert the intermediate to asymmetric dimethylarginine, the more common modification. In mammals, this class comprises PRMT1, -3, -4, -6, and -8 (2, 10). Due to sequence similarities, PRMT2 is regarded as a type I enzyme as well, but catalytic activity has not been demonstrated so far. Apart from PRMT8, which has been found in brain tissue and HEK 293 (T) cells only (2), type I PRMTs seem to be more or less ubiquitously expressed.

Arginine methylation is a group transfer reaction in which the methyl group is transferred from S-adenosylmethionine (AdoMet) to the guanidine moiety of the arginine side chain, presumably via an $S_2$ reaction (15). A reaction mechanism has been postulated based on structural evidence and mutagenesis experiments. The catalytic core, highly conserved between PRMTs, is formed by two domains. The N-terminal domain corresponds to the Rossmann fold and binds the methyl donor, as seen in co-crystals with the product S-adenosylhomocysteine (16–19). S-Adenosylmethionine is anchored in the active site by interactions with conserved glutamate and arginine residues. The C-terminal catalytic domain is a $\beta$-barrel structure. The methyl-accepting arginine side chain is bound between this domain and the S-adenosylmethionine binding domain, its guanidinium group being positioned via hydrogen bonds with two invariant glutamate residues; their mutation disrupts catalytic activity (18). Conceivably, these hydrogen bonds also fix the positive partial charge to one terminal nitrogen of the guanidino moiety and create a negative charge at the other, which is then ready to attack the methylsulphonium group of S-adenosylmethionine. Proton elimination may occur via a histidine-aspartate proton relay system (19). The PRMTs contain acidic grooves on the surface surrounding the active sites. These grooves seem to be able to bind arginine residues in the neighborhood of the one occupying the active site, as suggested by a co-crystal with a substrate peptide containing three methyl-accepting arginine residues. However, details of these interactions remain unknown, apparently due to the presence of several binding modes in the crystallized complex (18). As outlined above, monomethyl arginine is an intermediate in the reaction; addition of two methyl groups appears obliga-
Distributive Action of PRMTs

**Recombinant Proteins**—His-tagged PRMT1 was expressed and purified as described (30). His-tagged PRMT3 (30) was a kind gift of Silke Otto. Expression and purification of His-tagged PABPN1 and C-terminal deletion variants were performed as described (31). His-tagged hhnRNP K was a kind gift of Bodo Moritz. Protein concentrations were determined by UV spectroscopy using the theoretical absorption coefficients or by SDS-PAGE with subsequent Coomassie staining and bovine serum albumin as standard or by both methods.

**Synthetic Peptides**—Peptides were synthesized and analyzed as described (30). Sequences are shown in Fig. 1 and expected and detected masses in supplemental Table S1. The lyophilized peptides were dissolved in water. Their concentrations were determined by UV spectroscopy using the theoretical absorption coefficients.

**Methylation Assays**—Conditions were as published (30). Reactions were performed in a total volume of 20 μl and stopped by addition of SDS sample buffer and immediate freezing. If not stated otherwise, the initial concentration of [14C]-S-adenosylmethionine (GE Healthcare) was 85 μM, and the PRMT1 concentration was ~12 nM. For steady-state kinetic analysis, substrate proteins and peptides were titrated until saturation was achieved. The initial reaction rates were plotted directly against the substrate concentration and fitted to the Michaelis-Menten equation.

For MS analyses, conditions were as above except the total volume per assay was 210 μl, from which 20-μl samples were taken at the times indicated. The concentration of S-adenosylmethionine was typically 20 μM and that of PRMT1 varied between 30 and 300 nM, as indicated in the figure legends. Peptide concentrations were 36 μM (RXR-1 or -2, and RGG) and 50.9 μM (K2). The assays with K2 contained 200 μM S-adenosylmethionine. Assays with PRMT3 contained 0.21 μM enzyme, 200 μM S-adenosylmethionine, and 36 μM RGG, RXR-1, or RXR-2 peptide. For experiments with PABPN1, bovine serum albumin and Nonidet P-40 were omitted from the reaction buffer. These assays contained 12 μM PABPN1, 200 μM S-adenosylmethionine, and 0.3 μM PRMT1 or PRMT3. Reactions were started by the addition of S-adenosylmethionine (Sigma). After different time intervals 20-μl aliquots were taken, the reaction was stopped by addition of the methylation inhibitor sinefungin (Sigma) to a final concentration of 0.6 mM, the samples were frozen and later processed for mass spectrometry as described below. Similar assays containing the same concentration of [14C]-S-adenosylmethionine and peptides were performed in parallel. These samples were analyzed by SDS-polyacrylamide gel electrophoresis as described above.

**MALDI-TOF Mass Spectrometry**—Peptide samples were desalted and concentrated by C18 ZipTip® (Millipore); peptides were eluted with 75% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid in water. 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and
methylatable arginines are numbered according to the position in each peptide, arginine at position 10 in that peptide. The hnRNP K2 peptide derived from it. Arginine residues methylated in PABPN1 and hnRNP K isolated from cells many proteins. The amino acid sequence representing the methylated domain of hnRNP K (35) is aligned with C18, 300 were concentrated on a trapping column (Acclaim PepMap nanoelectrospray ionization source (Proxeon). The samples were acquired on an Ultraflex III MALDI-TOF/TOF mass spectrometer (ThermoFisher Scientific) equipped with a LTQ-Orbitrap XL hybrid system (LC Packings) coupled to an UltiMate Nano-HPLC (Roche Diagnostics). Proteolysis products were purified by reversed phase chromatography on an UltiMate Nano-HPLC (LC Packings) coupled to an LTQ-Orbitrap XL hybrid mass spectrometer (ThermoFisher Scientific) equipped with a nanoelectrospray ionization source (Proxeon). The samples were concentrated on a trapping column (Acclaim PepMap C18, 300 μm × 5 mm, 5 μm, 100 Å, LC Packings) and washed with 0.1% formic acid at 30 μl/min for desalting. After 15 min, peptides were eluted over the separation column (BioBasic-4, 75 μm × 15 cm, 5 μm, ThermoFisher Scientific), which had been equilibrated with 100% solvent A (5% acetonitrile, 0.1% formic acid). The gradient for separation was 0–100% B (80% acetonitrile, 0.1% formic acid) in 15 min, followed by 100% B for 45 min at a flow rate of 300 nl/min. The trapping column was washed with methanol for 30 min and re-equilibrated with 0.1% formic acid between the runs. Between each sample, a blank run was performed using the same gradient as for the samples. Online MS full scans in the Orbitrap analyzer were performed in the 400–2000 m/z range (resolution 60,000 at m/z 400). For each spectrum 17 microscans were accumulated. MS and HPLC were controlled by Xcalibur 2.0.7 (Thermo Scientific) with DCMSlink 2.0 (Dionex). Data processing was performed with the Xcalibur Qual Browser. All mass spectra recorded during the elution of the C-terminal Lys-C fragment of PABPN1 were averaged and deconvoluted using the Xtract tool.

Fluorescence Measurements—Fluorescence emission measurements were done with Fluoromax-2 or -3 fluorescence spectrophotometers (Horiba Jobin Yvon) in a fused silica cuvette at 30 °C in 50 mM K2HPO4/KH2PO4, 50 mM KCl, pH 7.5, in the presence or absence of 36 μM S-adenosylhomocysteine or 40 μM S-adenosylmethionine. Protein and peptide concentrations are indicated in the figure legends. For equilibrium experiments, the complex was allowed to form for 10 min at 30 °C prior to measurement. Fluorescence emission spectra were recorded from 305 to 420 nm on excitation at 295 nm using a 5-nm slit width. The integration time was 1 s; each spectrum was an average of three single spectra. For kinetic measurements, 5 μM RXR-2 peptide or PABPN1 were incubated with 1 μM PRMT1 in the presence or absence of 36 μM S-adenosylhomocysteine or 40 μM S-adenosylmethionine. After complex formation for 10 min at 30 °C, an 8-fold excess of K2 peptide was added. The subsequent dissociation of RXR-2/PABPN1 from PRMT1 was followed by the loss of fluorescence intensity at 355 nm.

RESULTS
Using the methyl donor S-adenosylmethionine as well as a methyl-accepting protein, PRMTs catalyze a classic two-substrate reaction. Thus, the variation of both substrate concentrations provides insight into the reaction mechanism (33). Accordingly, the concentration of S-adenosylmethionine was varied at different concentrations of methyl acceptors. These experiments were carried out with PRMT1 and PRMT3 and two different methyl acceptors, PABPN1 and a standard PRMT substrate peptide, RGG (30) (Fig. 1). Both enzymes behaved in a similar manner with both substrates: any increase in peptide or protein substrate concentration accelerated the S-adenosylmethionine consumption and vice versa till saturation was reached. The linearized plots intersected in one point in the second quadrant (Fig. 2 and additional data not shown). These results are consistent with previous data on PRMT1 and -6 (see Introduction) and support a ternary complex mechanism (33).

For the investigation of PRMT processivity by product analysis, it was first necessary to determine whether substrates containing either monomethylated arginine or dimethylated arginine together with additional methylatable arginines were preferred substrates for PRMTs. Thus, corresponding synthetic peptides were compared as substrates at saturating S-adenosylmethionine concentrations. Initial reaction rates revealed a hyperbolic dependence on peptide concentration (data not shown), and the data were evaluated according to the canonical Michaelis-Menten algorithm. Due to the transfer of several methyl groups to a single peptide, mixtures of different substrates are expected to be present in the course of the reaction. Therefore, all kinetic parameters presented should be regarded as apparent constants. However, they are adequate for comparing different substrates.

First, it was tested whether the substitution of arginine residues in the peptide RXX-1 (30) (Fig. 1) by monomethylarginine...
or asymmetric dimethylarginine would improve further methylation by PRMT1. As shown below, RXR-1 methylation by PRMT1 occurs exclusively at Arg\textsuperscript{10}. Substitution of Arg\textsuperscript{10} by monomethylarginine (peptide RXR-4) provoked a small decrease of both k\textsubscript{cat} and K\textsubscript{m} (supplemental Fig. S1 and Table 1) and no significant change in catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}). Thus, a preferential formation of dimethylated arginine residues specifically from previously monomethylated ones can be excluded. The substitution of a neighboring arginine, Arg\textsuperscript{9}, by asymmetric dimethylarginine (peptide RXR-5) resulted in minor changes of k\textsubscript{cat} and K\textsubscript{m} and an insignificant increase in catalytic efficiency. When Arg\textsuperscript{12} was also changed, so that both arginines flanking the preferred methyl-accepting Arg\textsuperscript{10} were asymmetrically dimethylated (peptide RXR-6), no changes in substrate properties were apparent either (supplemental Fig. S1 and Table 1). We conclude that, at least for the peptide investigated, the methylation of one arginine residue does not affect the methylation of neighboring residues by PRMT1.

When either Arg\textsuperscript{8} and Arg\textsuperscript{10} (peptide RXR-7) or Arg\textsuperscript{10} and Arg\textsuperscript{12} (peptide RXR-8) were replaced by asymmetric dimethylarginine, k\textsubscript{cat} was reduced to one-tenth, and the K\textsubscript{m} values went up about 10-fold (supplemental Fig. S1 and Table 1). As a result, the catalytic efficiency of PRMT1 against these peptides was reduced to less than 1%. This was expected because the preferred methyl acceptor, Arg\textsuperscript{10}, was no longer available. The data show that methylation of this residue does not enhance methylation of neighboring arginines. In summary, our data obtained with partially methylated RXR-peptides demonstrate that monomethylated arginine residues are not preferred substrates and that even the presence of two pre-methylated arginine residues does not accelerate the methylation of the remaining one.

We also tested whether mass spectrometry could be used for a quantitative analysis of methylation. For this purpose, peptides were modified with unlabeled S-adenosylmethionine, and the extent of total methylation was analyzed from weighted relative intensities of the MALDI-TOF MS signals for the various methylated species compared with the signal for the remaining unmodified substrate. In parallel, methylation was measured under identical conditions with the same concentration of [\textsuperscript{14}C]-S-adenosylmethionine at two different specific activities. The time courses of methylation determined by both methods were very similar (see supplemental Fig. S2 for two examples). When mixtures of three synthetic peptides were analyzed, amounts of the unmethylated peptide (RXR-1) were under estimated by 20.3 \pm 3.8\%, amounts of the singly methylated peptide (RXR-4) were underestimated by 11.7 \pm 5.7\%, and amounts of the 4-fold methylated peptide (RXR-6) were overestimated by 31 \pm 14.9\%. The magnitude of these deviations can be tolerated for the analyses described below. Moreover, because the proportions of multiply methylated products were small, the actual data accuracy was very likely better. The use of mass spectra for quantitative kinetic measurements has also been justified by others (34).

Processivity was then addressed by an analysis of the progress of methylation of several peptides by mass spectrometry. Peptides (Fig. 1) were incubated with PRMT1 under standard reaction conditions at an up to 100-fold molar excess of peptide over enzyme, and samples were taken after different time intervals and subjected to MALDI-TOF mass spectrometry. The relative peak integrals of the different methylated species were plotted against the incubation time. Furthermore, selected samples were investigated by MS-MS to establish the initial and subsequent methylation sites. The methylation sites found are marked in Fig. 1.

With peptide RXR-1, a single mass peak corresponding to one methylation event was observed at early time points, and a minor peak representing a second methylation event became apparent only later. Additional methylation events were not observed (Fig. 3A and supplemental Fig. S3). After a reaction time of 5 min, \textasciitilde 4 pmol of methyl groups had been incorporated per pmol of enzyme, in agreement with the independently determined k\textsubscript{cat} (\textasciitilde 0.01 s\textsuperscript{-1}). Even though several turnovers had taken place, there was a 5-fold excess of singly methylated compared with doubly methylated products. Thus, methylation was distributive. The monomethylated peptide remained the dominant product over the entire incubation time. The experimentally determined fractions of unmethylated, mono- and dimethylated peptides over the entire time course were compared with those predicted by a random (Poisson) distribution from the total number of methyl groups incorporated. Throughout

### TABLE 1

| peptide       | V\textsubscript{max} (pmol min\textsuperscript{-1} mg\textsuperscript{-1}) | k\textsubscript{cat} (s\textsuperscript{-1}) | K\textsubscript{m} (M) | k\textsubscript{cat}/K\textsubscript{m} (M\textsuperscript{-1} s\textsuperscript{-1}) |
|---------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------------------------|
| K\textsuperscript{2} | 20.670                      | 1.5 \times 10\textsuperscript{-2} | 0.09                        | 168.0                                           |
| RGG\textsuperscript{a} | 18.220                      | 1.3 \times 10\textsuperscript{-2} | 2.77                        | 4.3                                            |
| RXR-1\textsuperscript{a} | 36.020                      | 2.5 \times 10\textsuperscript{-2} | 1.25                        | 20.1                                           |
| RXR-2\textsuperscript{a} | 26.100                      | 1.8 \times 10\textsuperscript{-2} | 0.33                        | 54.9                                           |
| RXR-4\textsuperscript{a} | 17.770                      | 1.2 \times 10\textsuperscript{-2} | 0.69                        | 17.9                                           |
| RXR-5 | 25.720                      | 1.8 \times 10\textsuperscript{-2} | 0.61                        | 29.5                                           |
| RXR-6 | 30.520                      | 2.1 \times 10\textsuperscript{-2} | 0.70                        | 30.4                                           |
| RXR-7 | 3.655                       | 0.24 \times 10\textsuperscript{-2} | 22.24                       | 0.1                                            |
| RXR-8 | 3.889                       | 0.27 \times 10\textsuperscript{-2} | 8.01                        | 0.3                                            |

\textsuperscript{a} Data from Ref. 30.

\[ \frac{1}{v} = \frac{k_{\text{cat}}}{K_{\text{m}}} + \frac{1}{V_{\text{max}}} \]

\[ 1/[\text{SAM}] = \frac{k_{\text{cat}}}{V_{\text{max}}} + \frac{1}{V_{\text{max}} K_{\text{m}}} \]

\[ \frac{1}{v} = \frac{k_{\text{cat}}}{K_{\text{m}}} + \frac{1}{V_{\text{max}}} \]

\[ 1/[\text{SAM}] = \frac{k_{\text{cat}}}{V_{\text{max}}} + \frac{1}{V_{\text{max}} K_{\text{m}}} \]
Distributive Action of PRMTs

By MALDI-TOF/TOF-MS/MS analysis of the RXR-1 peptide, both methyl groups were found to be added to Arg10 (data not shown). Because methylation was restricted to a single arginine residue, the results do not rule out that multiple arginines in a single peptide can be methylated in a processive manner. Peptide RXR-2 was methylated at more than one residue: in the MALDI spectra of later time points of RXR-2 methylation, three signals were seen (supplemental Fig. S4). The first single methylation was found at Arg15 and the second, less pronounced one, at Arg18 by MS-MS (data not shown). As with the previous two peptides, the distribution of the three species was random, demonstrating a distributive mechanism (Fig. 3C).

Peptide K2, derived from the protein hnRNP K, is methylated by PRMT1 with a very high apparent affinity (30) and thus had the highest probability of being methylated processively. The four arginine residues contained in this peptide correspond to arginines 296, 299, 303, and 305 in hnRNP K. In the protein, Arg296 and Arg299 are quantitatively dimethylated, Arg303 is partially dimethylated, and Arg305 is unmethylated (35). Four different methylated peptides could be detected (supplemental Fig. S5). Surprisingly, these corresponded to monomethylation at all four arginines, as determined by MS/MS; even when the unmethylated peptide was almost depleted, no dimethylated product was detected. As with the other peptides, monomethylated peptide remained the dominant modified species over an incubation time of 30 min encompassing many methylation events per enzyme molecule, and the experimentally determined fractions of the different methylated species was in reasonable agreement with the predictions of a random distribution (Fig. 3D). Thus, methylation of the K2 peptide was distributive. Similar data were obtained at different concentrations of peptide and S-adenosylmethionine.

To confirm the distributive methylation of peptides by PRMT1, a substrate challenge experiment was carried out. Methylation by [14C]S-adenosylmethionine was measured by SDS-gel electrophoresis. Peptide RXR-3 (Fig. 1) was used as a competitor, because its 14C-labeled product could be distinguished from the product of K2 methylation. A 17-fold excess of RXR-3 over K2 was sufficient to block methylation of K2 essentially completely (Fig. 4 and supplemental Fig. S6). Methylation of K2 by PRMT1 was initiated in two identical reaction mixtures, and the rate of methyl incorporation was determined over a period of 12.5 min. Then, a 17-fold excess of RXR-3 was added to one reaction. As a result, further methylation of K2 was largely blocked, whereas K2 methylation proceeded in the control reaction, which received no competitor peptide (Fig. 4 and supplemental Fig. S6). Addition of one additional methyl group per enzyme to the previously bound substrate after the

FIGURE 3. PRMT1 methylates peptide substrates distributively. A, methylation time course of 38 μM RXR-1 by 0.4 μM PRMT1 in the presence of 19 μM S-adenosylmethionine. B, methylation time course of 37 μM RXR-2 by 0.27 μM PRMT1 in the presence of 19 μM S-adenosylmethionine. C, methylation time course of 36 μM RGG by 0.28 μM PRMT1 in the presence of 17 μM S-adenosylmethionine. D, methylation time course of 51 μM K2 by 0.23 μM PRMT1 in the presence of 210 μM S-adenosylmethionine. Methylation was analyzed by mass spectrometry as described under “Experimental Procedures.” The relative abundance of unmethylated (○), monomethylated (□), and dimethylated (△) peptide is compared with the theoretically predicted relative abundance of unmethylated (●), monomethylated (■), and dimethylated (▲) peptides. Predictions were based on the Poisson distribution.

the time course, the experimentally determined fractions of the three species were in good agreement with a random distribution (Fig. 3A). Random incorporation of methyl groups proves a distributive mechanism. In this assay, total methyl group incorporation at later times may have been limited by the relatively low concentration of S-adenosylmethionine used and the correspondingly high relative concentration of the accumulated product S-adenosylhomocysteine. However, this does not concern early time points and, thus, does not affect the conclusion of distributive activity.

The RGG-peptide is a rather weak substrate of PRMT1 (30). Again, two methylation signals could be detected (supplemental Fig. S4). The first single methylation was found at Arg3 and the second, less pronounced one, at Arg15 by MS-MS (data not shown). As with the previous two peptides, the distribution of the three species was random, demonstrating a distributive mechanism (Fig. 3C).

Peptide K2, derived from the protein hnRNP K, is methylated by PRMT1 with a very high apparent affinity (30) and thus had the highest probability of being methylated processively. The four arginine residues contained in this peptide correspond to arginines 296, 299, 303, and 305 in hnRNP K. In the protein, Arg296 and Arg299 are quantitatively dimethylated, Arg303 is partially dimethylated, and Arg305 is unmethylated (35). Four different methylated peptides could be detected (supplemental Fig. S5). Surprisingly, these corresponded to monomethylation at all four arginines, as determined by MS/MS; even when the unmethylated peptide was almost depleted, no dimethylated product was detected. As with the other peptides, monomethylated peptide remained the dominant modified species over an incubation time of 30 min encompassing many methylation events per enzyme molecule, and the experimentally determined fractions of the different methylated species was in reasonable agreement with the predictions of a random distribution (Fig. 3D). Thus, methylation of the K2 peptide was distributive. Similar data were obtained at different concentrations of peptide and S-adenosylmethionine.

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addition of competitor would have been within the error of the measurement. However, addition of a second methyl group per enzyme would have been clearly detectable and can be excluded. This result limits the processivity to a maximum of two methyl groups incorporated per binding event with this substrate.

Finally, the stability of a PRMT1-peptide complex was determined by fluorescence spectroscopy. When the K2 peptide, which lacks fluorescent groups, was added to PRMT1, enzyme fluorescence did not change (data not shown). However, when a tryptophan-containing substrate (peptide RXR-2) was used, an increase of the fluorescence signal was detectable upon binding to the enzyme (Fig. 5A). As this increase was rather weak, the total fluorescence signal was dominated by the free partners, and equilibrium dissociation constants could not be determined very reliably. Upon addition of K2 to the preformed PRMT1-RXR-2 complex, fluorescence intensity decreased in an apparently biphasic manner. The slower phase was also observed when K2 was not added; thus, it was assumed to be due to photobleaching and not considered further. The faster phase occurred only when K2 was added to the complex and was assumed to represent the dissociation of the PRMT1-RXR-2 complex (Fig. 5B). Independently of the presence or absence of S-adenosylmethionine or S-adenosylhomocysteine, dissociation rate constants in the range of ~0.02 s\(^{-1}\) were found (Table 2). The similarity of the dissociation rate constants to \(k_{\text{cat}}\) (Table 1) even in the presence of S-adenosylmethionine precludes multiple turnovers, thus providing additional evidence for a distributive mechanism.

PRMT3-catalyzed methylation of peptides RGG, RXR-1, and RXR-2 was also followed by mass spectrometry. Modification of RXR-1 and RXR-2 was each limited to the incorporation of a single methyl group. Two methyl groups were incorporated into peptide RGG, and this followed a random distribution, indicating a distributive activity (data not shown).

The data presented so far show that PRMT1 and PRMT3 act distributively on peptide substrates. However, it was conceivable that arginine residues in these peptides are not sufficiently distant from each other to simultaneously occupy different active sites in a PRMT oligomer. In contrast, multiple methyl-accepting arginine residues in natural proteins are often spread over larger distances, for example, 13 arginine residues in PABPN1 spanning a distance of 40 amino acids. Such substrates might be modified in a processive manner not seen with peptide substrates. In this case, the efficiency of methylation should be strongly dependent on the number and distance of methyl-accepting arginines. Therefore, the methylation of full-length PABPN1 was compared with that of several C-terminal deletion variants removing increasing portions of the methylated domain (Fig. 1). This depletion of substrate arginines led to a corresponding steep decrease in the efficiency of methylation.
Distribution of PRMTs

| Substrate | Cosubstrate/-product | \( k_{\text{off}} \) s\(^{-1} \) |
|-----------|----------------------|------------------|
| RXR-2     | S-Adenosylhomocysteine | 0.022 ± 0.011    |
| RXR-2     | S-Adenosylmethionine  | 0.019 ± 0.007    |
| PABPN1    | S-Adenosylhomocysteine | 0.031 ± 0.019    |
| PABPN1    | S-Adenosylmethionine  | 0.029 ± 0.015    |
| PABPN1    | S-Adenosylmethionine  | 0.016 ± 0.014    |

The standard error of \( V_{\text{max}} \) did not exceed 6%; that of \( K_m \) was typically below 13%.

| Protein     | \( V_{\text{max}} \) pmol min\(^{-1}\) mg\(^{-1}\) | \( k_{\text{cat}} \) s\(^{-1}\) | \( K_m \) \( \mu \text{M} \) | \( k_{\text{cat}}/K_m \) \( 10^5 \text{ s}^{-1} \text{ M}^{-1} \) |
|-------------|---------------------------------|-----------------|-------------|-----------------|
| PABPN1\(^a\) | 25.230                          | 1.8 × 10\(^{-2}\) | 0.07        | 270.0           |
| PABPN1ΔC8   | 33.344                          | 2.3 × 10\(^{-2}\) | 0.18        | 133.8           |
| PABPN1ΔC20  | 10.346                          | 7.2 × 10\(^{-3}\) | 0.37        | 18.9            |
| PABPN1ΔC27  | 2.469                           | 1.7 × 10\(^{-4}\) | 0.07        | 26.5            |
| PABPN1ΔC33  | 294                             | 2.1 × 10\(^{-4}\) | 0.03        | 6.7             |
| PABPN1ΔC40  | 603                             | 4.2 × 10\(^{-4}\) | 3.23        | 0.1             |

\(^a\) Data from Ref. 30.

by PRMT1 (supplemental Fig. S7 and Table 3). Whereas there was no clear-cut dependence of the apparent \( K_m \) values on the number of arginine residues, the apparent \( k_{\text{cat}} \) values were severely reduced with the shorter substrates (Table 3). A processive reaction mechanism would be one possible explanation for this effect.

As a direct assay of processivity, the methylation of PABPN1 by PRMT1 was investigated by ESI-MS of PABPN1 samples taken after different intervals of incubation with AdoMet and PRMT1. The samples were digested with the protease Lys-C, which cleaves off the entire C-terminal domain as a single peptide (see Fig. 1 for the amino acid sequence), and methylation was analyzed by LC/ESI-MS. Several differentially methylated species were observed; products containing up to four methyl groups were sufficiently above baseline for quantification (supplemental Fig. S8). As with the peptide substrates described above, monomethylated products were the dominant product species over the first 20 min of the time course, encompassing ~16 methylation events per enzyme molecule. Over the first 30 min, the distribution of the various methylated species was in reasonable, although not perfect agreement with a random distribution, supporting a distributive action of PABPN1 even on this substrate (Fig. 6). Only at later time points, when a significant fraction of the substrate had been methylated at least once, did the experimentally determined distribution differ from the prediction.

As a second test of processivity, a substrate challenge experiment with \(^{14}\)C-S-adenosylmethionine was done. In this case, methylation of PABPN1 by PRMT1 was allowed to proceed for 15 min before a sufficient amount of the competing substrate RXR-1 was added to quench further methylation of PABPN1. The subsequent addition of methyl groups to the first substrate was clearly less than 0.5/PRMT1 molecule. Thus, the reaction was distributive (Fig. 7 and supplemental Fig. S9). Similar results were obtained with PRMT3 (supplemental Fig. S9).

Binding of PABPN1 to PRMT1 was also associated with an increase in fluorescence, presumably due to the single tryptophan residue of PABPN1 close to its C terminus. The kinetic stability of a preformed PABPN1-PRMT1 complex was determined by the loss of fluorescence upon displacement of PABPN1 by an excess of K2 peptide (supplemental Fig. S10). As with the RXR peptide, \( k_{\text{off}} \) was very similar to \( k_{\text{cat}} \) and independent of the presence of S-adenosylmethionine or S-adenosylhomocysteine (Table 2). This strongly supports a distributive mechanism.

![FIGURE 6. PABPN1 is methylated distributively by PRMT1. 12 μM PABPN1 was methylated by 0.3 μM PRMT1 in the presence of 238 μM AdoMet, digested proteolytically, and analyzed by LC/ESI-MS as described under “Experimental Procedures.” The signal intensities in ESI mass spectra of unmethylated (○), monomethylated (□), and dimethylated (△) C-terminal fragments were normalized to the total signal intensities and compared with the relative abundance predicted by a Poisson distribution for unmethylated (●), monomethylated (■), and dimethylated (▲) fragments. Significant amounts of peptides containing more than two methyl groups were observed only upon longer incubation. The data presented were averaged from two independent experiments.]

DISCUSSION

The addition of two methyl groups to a single arginine side chain and the addition of methyl groups to multiple arginine side chains in a single protein or peptide can, in principle, occur in a distributive manner (i.e. with release of the substrate after each methylation event) or in a processive manner (i.e. with two or more methylation events occurring before dissociation of the substrate). Crystal structures of PRMT4 showed that part of the polypeptide chain moves upon S-adenosylmethionine binding, burying the co-substrate and at the same time forming part of the peptide binding pocket (17). This argues very strongly that release of the peptide is necessary to allow the exchange of S-adenosylhomocysteine for S-adenosylmethionine in preparation for a second round of methylation. Such an ordered mechanism seems incompatible with processive methylation. However, the structural argument refers to a single active site and does not take into account binding of a peptide at a second site. This second site could be, for example, an acidic groove on the same polypeptide (18) that interacts with a basic patch, distinct from the methyl-accepting arginine, on the peptide substrate (22). Thus, the random mechanism determined for PRMT1 by Thompson and co-workers (21) might be reconciled with the
FIGURE 7. PABPN1 can be displaced from PRMT1 by excess RXR-1 peptide. PRMT1-catalyzed methylation of 1.6 μM PABPN1 alone (●) proceeded linearly with time. The additional presence of 150 μM RXR-1 strongly suppressed methylation of PABPN1 (■) in favor of RXR-1 methylation (▲). In the time interval (15 min) before addition of RXR-1, PABPN1 methylation (▲) proceeded as usual and was quenched by RXR-1, which was then methylated exclusively (△). PABPN1 methylation is shown in A, RXR-1 methylation is shown in B. Raw data are shown in supplemental Fig. S9.

**Distributive Action of PRMTs**

Methyl transfer can be assessed in a number of ways. A distributive reaction will result in a random distribution of methyl groups over the entire time course of the reaction. In contrast, a processive reaction will result in an overrepresentation of products containing multiple methyl groups as compared with singly methylated or unmethylated substrates. The most direct assay of the processivity of a reaction consists of a determination of the number of catalytic events per binding event. In the simplest case, this can be done by providing a sufficient excess of substrate over enzyme and measuring the number of catalytic events per substrate, in this case the number of methyl groups incorporated per peptide or protein. A distributive reaction will result in a random distribution of methyl groups over all substrates. In contrast, a processive reaction will result in an overrepresentation of products containing multiple methyl groups as compared with singly methylated or unmethylated substrates.

With peptide RXR-1, which was methylated only at one of its three arginine residues, monomethyl arginine was the preferred product over the entire time course, and dimethyl arginine was underrepresented. The proportions of these two products and the unmethylated peptide very closely matched a random distribution over the entire time course of the reaction. The question was not pursued experimentally for peptides RXR-2, RGG, and K2, but with RXR-1, no discrimination against monomethylated products was seen.

Mass spectrometry can determine the proportions of the unmethylated substrates and the various methylated products only with modest accuracy. However, because a multiply methylated derivative of peptide RXR-1 was overrepresented, MS analysis tends to overestimate the processivity of the methylation reaction. Despite this bias, the MS data clearly support a distributive activity of PRMT1.

Remarkably, in both the RGG and the K2 peptide two distinct monomethylation events were preferred over the dimethylation of a single arginine. This is also strong evidence against processive methylation of a single arginine. It is also interesting to note that the methylation pattern of peptide K2 did not match the in vivo methylation pattern of the hnRNP K protein from which it was derived. Thus, either additional features of the protein or additional factors have an influence on the pattern of methylation.

Distributive action of PRMT1 on peptide substrates was confirmed by two additional experimental approaches. Further methylation of a pre-bound substrate peptide was quenched immediately upon the addition of a competing substrate, and k_{cat} and k_{off} determined by fluorescence measurements, was very similar to k_{cat} and k_{off} even under reaction conditions, i.e. in the presence of S-adenosylmethionine. The similarity of the two rate constants would be consistent with a mechanism in which substrate release is obligatory and rate-limiting for the addition of a further methyl group.

All three types of experiments were repeated with a natural substrate protein, PABPN1, which carries 13 methyl-accepting arginine residues spanning a distance of 40 amino acids. Thus, if a processive activity were dependent on the oligomeric structure of PRMTs enabling the enzymes to act on several arginine residues simultaneously, PABPN1 should have been a suitable substrate to reveal this behavior. However, as with peptide substrates, the distribution of methyl groups incorporated was random, a competing substrate rapidly quenched methylation of pre-bound PABPN1, and fluorescence measurements revealed a k_{off} that was similar to k_{cat} and also similar to k_{off} values obtained for a peptide substrate. Thus, even the methylation of multiple arginine residues spread over a considerable region of a protein substrate proceeds distributively. Deviations from a random distribution were observed upon longer incubation. An example can be seen in the lower panel of supplemental Fig. S8. This may reflect substrate preferences of the enzyme and pos-

structural evidence for an ordered mechanism. Alternatively, or in addition, the second binding site for the protein substrate could be the active site of another subunit in a PRMT oligomer, which simultaneously methylates a second arginine. Such a mechanism would provide a rationale for the oligomeric structures of PRMTs and the occurrence of clusters of dimethylarginines in most methylated proteins.

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Three additional peptides, RXR-2, RGG, and K2, were methylated at more than one arginine side chain. Nevertheless, the distribution of methylation events was clearly random in all three cases. We have not tested whether PRMT1, with these peptides, has a preference for re-binding of monomethylated reaction products. However, such a preference would make a distributive reaction seem processive; therefore, this behavior can be excluded. A discrimination against monomethylated products, which might make a partially processive reaction seem distributive, does not seem very likely in view of the close match between the experimentally determined and the calculated distribution. The question was not pursued experimentally for peptides RXR-2, RGG, and K2, but with RXR-1, no discrimination against monomethylated products was seen.

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Distributive Action of PRMTs

...ably also less accurate measurements of the abundance of multiply methylated proteins.

Incremental deletion of the arginine residues in the C terminus of PABPN1 leads to a progressive reduction of $k_{\text{cat}}$. As even the full-length protein is methylated distributively, a loss of processivity clearly cannot be the basis of this effect. One possible explanation would be the removal of preferred methylation sites, and in fact we have some preliminary evidence for this.

Some of the key experiments were repeated to examine the processivity of the methyltransferase PRMT3. The results were very similar to those obtained with PRMT1; thus PRMT3 also acts distributively on all substrates tested.

A processive enzyme can proceed faster than a distributive one because re-association with substrate after its dissociation is time consuming. For the same reason, a processive enzyme is faster with a substrate on which it can act multiple times as compared with one on which it can act only once or a few times. With PRMTs, very similar $k_{\text{cat}}$ values ($\sim 0.02 \text{ s}^{-1}$) were determined for a large number of substrates, including peptide RXR-1, which is methylated only on a single arginine residues, and PABPN1, which is methylated on multiple arginine residues (Tables 1 and 3) (30). These uniform $k_{\text{cat}}$ values are in agreement with a distributive reaction mechanism.

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