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Design, total chemical synthesis, and binding properties of a [Leu-91-N^1-methyl-7-azaTrp]Ras-binding domain of c-Raf-1

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ABSTRACT **The Ras-binding domain (RBD) of c-Raf-1 has been synthesized chemically, taking advantage of the chemical ligation of two peptide fragments of the protein. This procedure allowed incorporation of an unnatural amino acid (N^1-methyl-7-azatryptophan) at position 91 of RBD, producing a protein with fluorescent properties distinct from and distinguishable from those of proteins containing the natural fluorophore tryptophan. The resulting protein was shown to interact with Ras in a manner that was almost indistinguishable from that of unmodified RBD based on transient kinetic monitoring of the binding event. Modified RBD containing the L-isomer of the unnatural amino acid or its racemic D,L mixture appeared to interact identically with Ras. The approach demonstrates a general procedure for the introduction of unnatural amino acids that can be used for monitoring protein–protein interactions and for the introduction of an unnatural backbone structure at strategic positions.**

Deciphering protein–protein interactions is one of the challenging tasks in the elucidation of intracellular signal transduction pathways. In these molecular cascades the input signal is transferred from a receptor molecule to an effector protein, which, in turn, can stimulate or inhibit the activity of a wide variety of cellular proteins. All of these reactions involve the transient association of proteins, thereby promoting such processes as phosphorylation, methylation, or dissociation of heteromers like those of the G-protein family.

A central role in cell signaling events is played by proteins of the superfamily of small GTPases. For example, Ras relays signals from cell surface receptors to the nucleus to stimulate cell proliferation and differentiation (1). It functions as a switch between the inactive GDP-bound and the active GTP-bound form. In the latter activated state, Ras stimulates downstream effectors, in particular c-Raf-1 (2, 3). This interaction is of utmost importance for the regulation of cell division and differentiation. As a consequence, some mutations in Ras leading to inhibition of GTP hydrolysis or acceleration of GDP release are involved in about 30% of human cancers (4).

Previous studies have shown that a sequence of about 80 aa in the N-terminal region of c-Raf-1, the so-called Ras-binding domain (RBD), is sufficient for the interaction with Ras (5). This finding has led to extensive investigations of the interaction between Ras and the RBD protein using kinetic and structural methods (6–8). Structural as well as kinetic methods provide the means to analyze these interactions at the molecular level. Although there are only a few structures of signaling pathway protein–protein complexes published so far, a high-resolution RBD-Rap1A structure is available, with Rap1A as a member of the Ras family (6).

Detailed kinetic analysis of protein–protein interactions is not always possible because of the lack of suitable molecular monitors. This problem can be solved by site-specific introduction of a label that is able to detect the binding event without perturbing the kinetics of the process. In principle, the indole side chain of tryptophan can fulfill these requirements and can be used as a fluorescent probe to study protein–protein interactions. However, because of its small extinction coefficient and its poor quantum yield, the Trp residue displays only weak fluorescence and is not very sensitive to environmental effects. Furthermore, proteins often contain more than one Trp residue, making it difficult to assign the signal to a particular site or to separate a small change against a large static background. These difficulties can be partially overcome by either using extrinsic reporter groups, e.g., a coumarin derivative such as that which has been attached to the phosphate binding protein from *Escherichia coli* via a linker (9, 10) or by site-directed modification of the protein with an unnatural fluorophore. The latter procedure combines the advantages of an intrinsic fluorophore, which is located at a defined position in the modified protein with those of extrinsic reporter groups whose properties can be tuned to the particular problem under question.

An alternative, superior approach would be to incorporate unnatural fluorescent amino acid(s) directly into the polypeptide chain to probe protein–protein interactions with minimum perturbation of the system. Initial attempts to use conventional molecular biological techniques to label proteins in this way already have been described. For example, tryptophan analogs such as 5-hydroxytryptophan and 7-azatryptophan were incorporated successfully into proteins in Trp auxotrophic expression systems (11–13). However, this method is restricted to a few analogs that can replace Trp successfully in the biosynthetic machinery of a cell. Furthermore, 100% incorporation is not ensured and the method is restricted to proteins containing at most only a few tryptophans, because all residues are replaced by its analog. Another potentially very far-reaching possibility has been designed by Schultz and coworkers (14), who developed an *in vitro* suppression system that allowed the site-directed modification of proteins by a wide variety of different α-amino acids. In this way 7-azatryptophan was introduced into T4 lysozyme, and its fluorescence spectrum was obtained (15). Despite the ability of *in vitro* suppression to incorporate site, specifically a wide variety of unnatural amino acids, the overall yields of protein synthesis unfortunately are still quite low. Furthermore, upscaling of the experiments

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Abbreviations: Boc, *tert*-butyloxycarbonyl; GppNHp, guanyl-5′-yl-imidodiphosphate; L91L-O, protein analogue with leucine at position 91 replaced by the L-enantiomer of N^1-methyl-7-azatryptophan (other analogues are abbreviated correspondingly); mant, 2′,3′-(*N*-methylanthraniloyl); RBD, Ras-binding domain; RBD/H, RBD with an additional His tag at the C terminus.

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remains difficult, rendering the subsequent biophysical analysis of the site-mutated probes difficult if not impossible.

As an alternative to these molecular biology approaches, total chemical protein synthesis would in principle allow complete versatility in the precise introduction of probe moieties at any desired site(s) in the protein molecule. Recent innovations, most notably the reaction of large unprotected synthetic peptides by amide-forming ligation at Cys residues (16–18), have rendered feasible the routine, reproducible preparation of a wide range of proteins of up to ≈20 kDa in good amounts (19, 20). Chemically synthesized proteins can readily be obtained in multimilligram amounts and in a purity sufficient for determination of high-resolution structures by x-ray crystallography (21, 22) and for study by NMR and other biophysical techniques (23).

In this paper, we describe the design of an RBD protein that contains the artificial amino acid N^1-methyl-7-azatryptophan at a predetermined location to probe the interaction with Ras. The efficient total chemical synthesis of the unnatural protein is described, together with the characterization of its Ras-binding properties by stopped-flow fluorescence measurements. These data demonstrate the feasibility of an approach that has broad potential application to the study of protein–protein interactions.

MATERIALS AND METHODS

Materials. Tert-butyloxycarbonyl (Boc)-protected amino acids were purchased from Peptides International. 7-Azatryptophan was bought from Sigma. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate was obtained from Spectrum (Gardena, CA). The Boc-amino acyl-OCH_2-phenylacetamidomethyl resins were bought from Applied Biosystems. *N*,*N*-diisopropylethylamine was obtained from Applied Biosystems, and trifluoroacetic acid was from Halocarbon Products (Hackensack, NJ). Hydrogen fluoride was purchased from Matheson.

Synthesis of N^1-Methyl-7-Azatryptophan. The fluorophore was synthesized as reported by Rich *et al.* (24), and the Boc-protected enantiomers were separated by HPLC-enantioseparation with Teicoplanin as the chiral stationary phase (25, 26). According to those authors, L-enantiomers elute first, an observation that allowed the configurational assignment of L-Boc-N^1-methyl-7-azatryptophan.

Peptide Synthesis. Solid-phase peptide synthesis (27) was performed manually or on a custom-modified 430A peptide synthesizer from Applied Biosystems, using *in situ* neutralization/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate activation protocols for stepwise Boc chemistry chain elongation (28). The N-terminal peptide fragments were synthesized on a thioester-generating resin (29). The coupling of Boc-protected N^1-methyl-7-azatryptophan was achieved by activation using the 1-hydroxy-7-azabenzotriazole/diisopropylcarbodiimide method. After HF cleavage, the peptides were purified by RP-HPLC with a C4-column from Vydac (Hesperia, CA) by using linear gradients of buffer B (acetonitrile/0.1% trifluoroacetic acid) in buffer A (H_2O/0.1% trifluoroacetic acid) and UV detection at 214 nm. Samples were analyzed by electrospray mass spectrometry with a Perkin–Elmer Sciex API-I quadrupole mass spectrometer.

Chemical Protein Synthesis. Full-length polypeptide chains were synthesized by native chemical ligation (16–18) of the peptide fragments (2 mM) in 6 M GuHCl, 200 mM phosphate, pH 7, and 1% thiophenol. The reactions usually were carried out overnight. The resulting polypeptide products were purified and analyzed as described above for the peptide segments.

Folding of Proteins. The purified polypeptide chains of the RBD proteins (about 1 mg/ml) were dissolved in 100 mM NaCl, 50 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4, 5 mM DTT. The solution was gently stirred for 1 h at 8°C, after which the samples were shock-frozen in liquid nitrogen and stored at −80°C.

CD Spectroscopy. For CD spectroscopic measurements, the samples (95 μM) were dialyzed against 20 mM phosphate, pH 7.4. CD spectra were recorded on a Jasco J-710 spectropolarimeter at 20°C by using a quartz cell with 0.2-cm pathlength. The spectra were corrected for buffer contributions.

Fluorescence Spectroscopy. Fluorescence spectra were measured with an Aminco-Bowman Series 2 luminescence spectrophotometer at 25°C. The proteins (2 μM) were dissolved in 100 mM NaCl, 50 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4.

Stopped-Flow Measurements. The stopped-flow measurements were carried out as described (8). N^1-methyl-7-azatryptophan was excited at 313 nm, and the emitted light was monitored through filters with a cut-off wavelength of 360 nm.

RESULTS AND DISCUSSION

Protein Design. The activation of Ras effectors such as c-Raf-1 by the small GTPase Ras is a key reaction for the stimulation of cells by extrinsic signals. A malfunction in this step caused by a mutation often is involved in malignant transformation in human cells (4). It therefore is of great interest to elucidate the molecular mechanisms of the intermolecular signal transfer. The work described here concerns the production and use of monitors that permit the detection of the binding process without disturbing it. Ideally, the label used for this purpose should be susceptible toward environmental changes and should possess high intrinsic sensitivity. These properties can be fulfilled by various fluorescent dyes. However, in general, the more sensitive dyes, which are large in size, potentially could preclude perturbation-free detection of protein–protein interactions.

In the work presented here, we have used a fluorescent amino acid (N^1-methyl-7-azatryptophan), which combines a size similar to that of the naturally occurring Trp with considerably higher sensitivity (24). In previous work it has been shown that N^1-methyl-7-azatryptophan is stable toward the conditions of solid-phase peptide synthesis (24, 30) so that it also can be used in the synthesis of larger peptide fragments.

Having selected the optical probe, its site within the protein had to be assigned. An analysis of the known crystal structure of the RBD-Rap1A complex (6) revealed that in the RBD, the position of Leu-91 might fulfill the conditions for an intrinsic monitor of the protein–protein interaction (Fig. 1). This site is not directly involved in the binding process, but is close enough to the interacting region to potentially detect the binding of Ras. This rationale was verified in earlier experiments in which Leu-91 was replaced by Trp (8). It could be shown that Trp-91 of the RBD mutant can serve as an optical probe and hardly

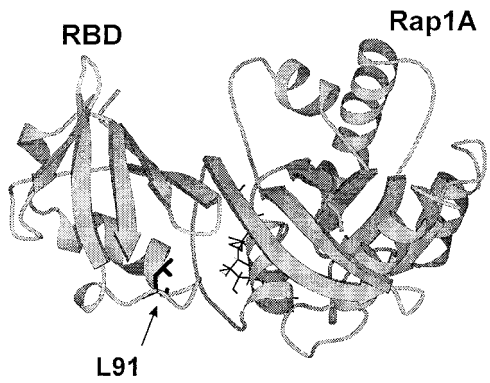

FIG. 1. Ribbon presentation of the Rap1A-RBD complex (6). Leucine 91 (L91) is shown as a stick model. Figure was drawn with MOLSCRIPT (39).

disturbs the actual binding process (see Table 1, reaction 7 for the kinetic data).

Another aspect of this work concerned the incorporation of D-amino acids into proteins. If the position in question is not essential for the biological activity and it is also not crucial for the structure of the target protein, then a D-amino acid in this position might be tolerated by the protein without disturbing its activity. In this case, the use of either a D- or L-enantiomer could provide another possibility to achieve a slight change in the properties of the label.

A third modification also was introduced into the RBD protein. A C-terminal His tag allowed a facile purification of native RBD, which had been biosynthetically prepared (8). To compare the chemically and biochemically synthesized proteins the His tag also was attached to the chemical synthesized RBD. Additionally, such a linker could serve to immobilize the protein on Ni^{2+}-surfaces for biofunctionalization (31).

According to the considerations discussed above, three RBD analogues were prepared: (*i*) RBD/H, which has the wild-type protein sequence with an additional His tag at the C terminus; this protein has exactly the same sequence as the RBD/H, which was prepared earlier by recombinant DNA technology (8) and serves as a reference molecule in the present work, (*ii*) [L91L-O]RBD/H, which contains in addition to the His tag L-N^1-methyl-7-azatryptophan instead of Leu-91, and (*iii*) [L91D,L-O]RBD/H, in which the D,L-racemate of the artificial fluorophore was incorporated at position 91.

Total Chemical Synthesis of the Proteins. The experimental strategy for the introduction of these modifications by total chemical synthesis is outlined in Fig. 2. In each case, two unprotected segments spanning the target polypeptide were synthesized by stepwise solid-phase synthesis (27, 32). In this case, we used the *in situ* neutralization/2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate activation protocols for Boc chemistry (28). The N-terminal segments, comprising amino acids 50–95, were synthesized on a resin designed to generate a thioester at the C terminus after HF cleavage (29). The C-terminal fragments, comprising amino acids 96–140 (including the His tag) were synthesized on a standard –OCH_2-phenylacetamidomethyl resin (33). Native chemical ligation of these segments was carried out at the Cys-95–Cys-96 sequence according to previously described procedures (16–18). The 91-residue polypeptide product was purified by RP-HPLC.

Fig. 3 shows as an example the final elution profile (Fig. 3*a*) and the electrospray mass spectrum (Fig. 3*b*) of [L91D,L-O]RBD/H. The mass spectrometry shows the $5H^+$ to $11H^+$ charged states of the purified polypeptide with a relative mass of 10,544.5 ± 1.1 Da (calculated mass with an average isotope composition: 10,544.1 Da), whereas the HPLC chromatogram reveals the high purity (>98%) of the chemically synthesized protein. The other two target polypeptides were obtained at a similar level of purity and with a recovered yield of about 25% based on the peptide segments.

CD and Fluorescence Spectroscopy. The lyophilized polypeptides first were reconstituted in the aqueous buffer (100 mM NaCl, 50 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4, 5 mM DTT as antioxidant), which was the same as that used for the stopped-flow experiments. The three polypeptides dissolved immediately at a final concentration of about 1 mg/ml. The far-UV CD spectra of the folded proteins are shown in Fig. 4*a* together with those of the biosynthetic RBD/H and [L91W]RBD/H. Generally, there is good agreement between the spectra and those previously published (34, 35). The fact that the chemically synthesized proteins adopt a tertiary structure similar to that of the native proteins almost immediately after dissolving in physiological buffer is an important prerequisite for subsequent experiments. This observation is another example of the widespread applicability of total chemical synthesis of proteins (23). Even chemically synthesized proteins with numerous disulfide bonds such as the secretory phospholipase A_2 can be readily reconstituted in their correctly folded, active form (20).

In Fig. 4*b* the fluorescence spectra of [L91L-O]RBD/H that contains the unnatural fluorophore and the corresponding Trp mutant [L91W]RBD/H are compared. The incorporation of N^1-methyl-7-azatryptophan leads to an increase of the emission intensity with a considerable red shift of the fluorescence maximum as compared with [L91W]RBD/H. This difference allows a selective detection of the emission for e.g., the study of protein–protein interactions even in the presence of responsive Trp residues.

Stopped-Flow Measurements. The properties of the chemically synthesized proteins were determined by stopped-flow techniques. In these experiments, Ras was loaded with the fluorescent nonhydrolysable GTP analogue 2′,3′-(*N*-methylanthraniloyl)guanyl-5′-yl-imidodiphosphate (mantGppNHp). Binding of RBD to this Ras complex results in a transient change of the mant fluorescence. The binding transients at the concentrations occur in the millisecond time range and therefore are well suited for stopped-flow investigations. The results of these experiments are compiled in Table 1 (reactions 1–4). In a second set of experiments the binding kinetics were measured by using fluorescence monitors located in the other reaction partner RBD (Table 1, reactions 5–7).

A comparison of the kinetic data of His-tagged RBD expressed in *E. coli* (Table 1; reaction 4) with those of RBD that had been prepared by chemically means (Table 1; reaction 1) showed no significant differences in the Ras-binding be-

Table 1. Kinetic parameters for the interaction of chemically synthesized and biosynthetic RBD proteins with H-Ras at 25°C

Reaction	$k_{ass}/M^{-1}\cdot s^{-1}$	k_{diss}/s^{-1}	K_d/nM
1. csRBD/H + H-Ras.mantGppNHp	$2\cdot10^7$	6.6	330
2. cs[L91D,L-O]RBD/H + H-Ras.mantGppNHp	$1.7\cdot10^7$	7.0	410
3. cs[L91L-O]RBD/H + H-Ras.mantGppNHp	$1.8\cdot10^7$	6.8	380
4. RBD/H + H-Ras.mantGppNHp†	$2.1\cdot10^7$	6.4	300
5. cs[L91D,L-O]RBD/H + H-Ras.GppNHp	$2.8\cdot10^7$	11.4*	400
6. cs[L91L-O]RBD/H + H-Ras.GppNHp	$3.4\cdot10^7$	10.3*	300
7. [L91W]RBD/H + H-Ras.GppNHp†	$3.6\cdot10^7$	14	380

Reactions 1–4: Kinetics monitored by using the mant-fluorescence of H-Ras.mantGppNHp. Reactions 5 and 6: Kinetics monitored by using the N^2-methyl-7-azatryptophan fluorescence of the artificial RBD proteins. The dissociation rates were obtained by displacement reactions as described (8). Reaction 7: Kinetics monitored by using the Trp-fluorescence of the [L91W]RBD/H mutant.

*Because of the slow second phase that sometimes also occurred in the displacement experiments we chose k_{obs1} for k_{diss}, because it correlates well with our previous results for the dissociation rate of the RBD-Ras interaction.

†As published in ref. 8.

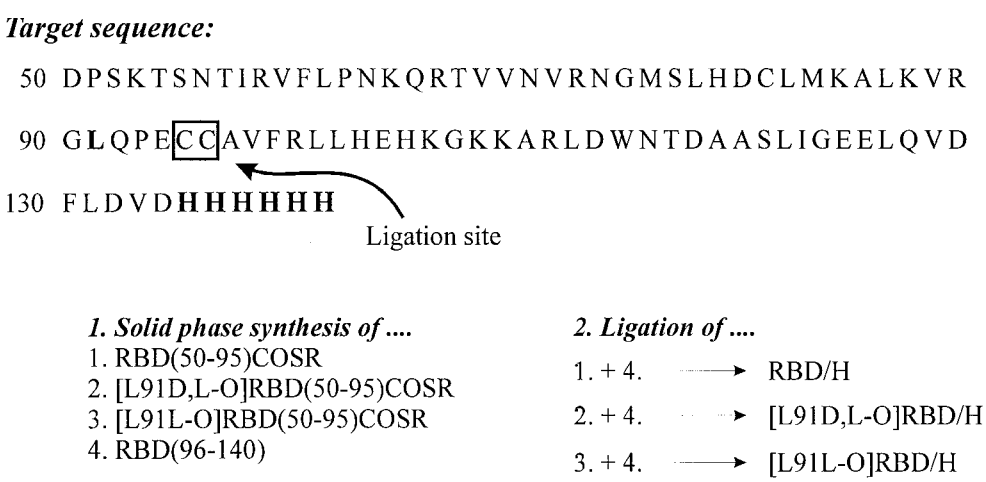

FIG. 2. Experimental strategy for the total chemical synthesis of RBD proteins. Two peptide segments of the target polypeptide are synthesized by optimized solid-phase synthesis protocols (28) and coupled by native chemical ligation at the ligation site Cys-95–Cys-96 (16, 18). Modifications are in bold. COSR, C-terminal thioester. O, optical probe, in this case N^1-methyl-7-azatryptophan, where D,L-O is the racemate and L-O is the L-enantiomer. Numbering is taken from the sequence of c-Raf-1.

havior. This observation is important, because it shows that the two methods of protein preparation lead to proteins with identical activity. The proteins containing the unnatural fluorophore, [L91D,L-O]RBD/H and [L91L-O]RBD/H, have only a slightly reduced affinity for Ras compared with RBD/H (Table 1; reactions 2 and 3). Furthermore, there is also no difference between the data of the L-enantiomer [L91L-O]RBD/H (Table 1; reaction 3) and those of the D,L racemic mixture [L91D L-O]RBD/H (Table 1; reaction 2). These results show that the incorporation of a D-amino acid into the polypeptide chain is not necessarily detrimental to a protein's folding or function. Obviously, the loop region at the position of Leu-91 is not vital to binding activity nor to the three-dimensional structure of RBD. This is an interesting point in the context of the stability of proteins toward proteases. There are already an increasing number of peptide drugs containing D-amino acids that show enhanced protease stability (36). The incorporation of D-amino acids into proteins without decreasing their activity but enhancing their stability might be of importance for the design of drugs with increased pharmacokinetic stability.

In further experiments, the response of N^1-methyl-7-azatryptophan at position 91 toward binding of RBD to Ras was analyzed. Fig. 5 shows the signal of this unnatural fluorophore on binding of unlabeled H-Ras.GppNHp. The Trp mutant [L91W]RBD/H shows no signal under the same conditions, which demonstrates that the original idea of a sensitive fluorescent label could be verified. The corresponding kinetic data in Table 1 (reactions 5 and 6) clearly indicate that the artificial chromophore has no influence on the binding kinetics as compared with the Leu-91–Trp mutant (Table 1; reaction 7). k_{ass} measured with the intrinsic chromophore in RBD is larger than that measured by using the fluorescent nucleotide signal. This result is in agreement with previous work, in which the methylanthraniloyl group was shown to have a slight effect on the association kinetics of Ras and RBD (8). Thus, the chromophore at position 91 appears to cause less disturbance in the association reaction than the modification of the nucleotide structure.

An interesting additional observation concerns the fluorescence changes on interaction of Ras and the [L91L-O]RBD/H and [L91D,L-O]RBD/H probes, which were indistinguishable under the conditions examined. Apparently, the chromophore although in slightly different environments in the D- and L-forms detects the same changes on the binding of Ras, an observation that has intriguing potential for the introduction of D-amino acid fluorophores into proteins. Nevertheless, it is to be expected that the incorporation of D-amino acids in defined positions generally will affect the structural and func-

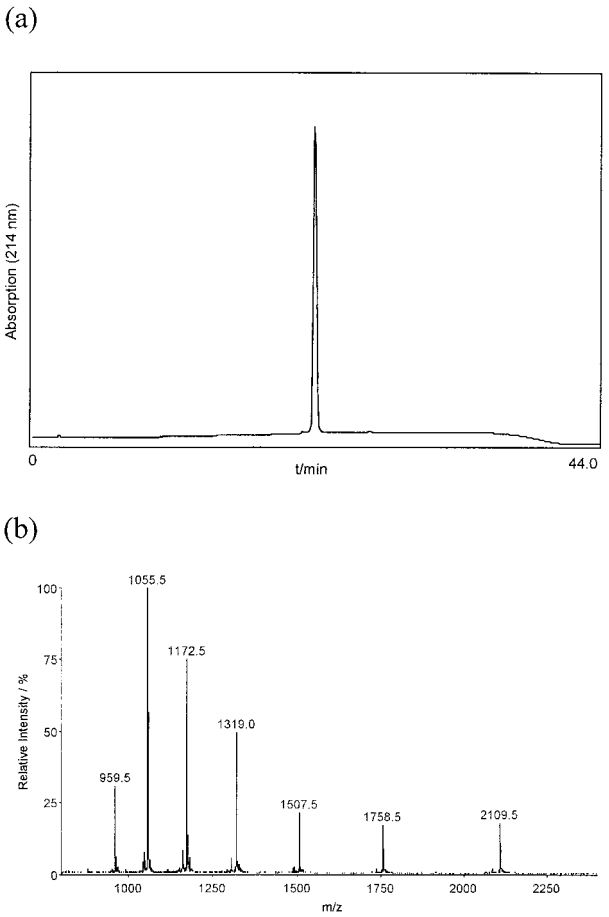

FIG. 3. Characterization of purified [L91D,L-O]RBD/H, the artificial protein with N^1-methyl-7-azatryptophan as a racemate at position 91 and a C-terminal His tag. (*a*) Chromatogram after purification on a preparative C4 RP-HPLC column. The chromatogram was obtained on an analytical C4 RP-HPLC column with a gradient of 5–65% acetonitrile in H_2O, 0.1% trifluoroacetic acid over 30 min. (*b*) Electrospray ionization-mass spectrometry of the purified product with the $5H^+$–$11H^+$ charged states of the polypeptide corresponding to a measured mass of 10,544.5 ± 1.1 Da (calculated mass with an average isotope composition: 10,544.1 Da).

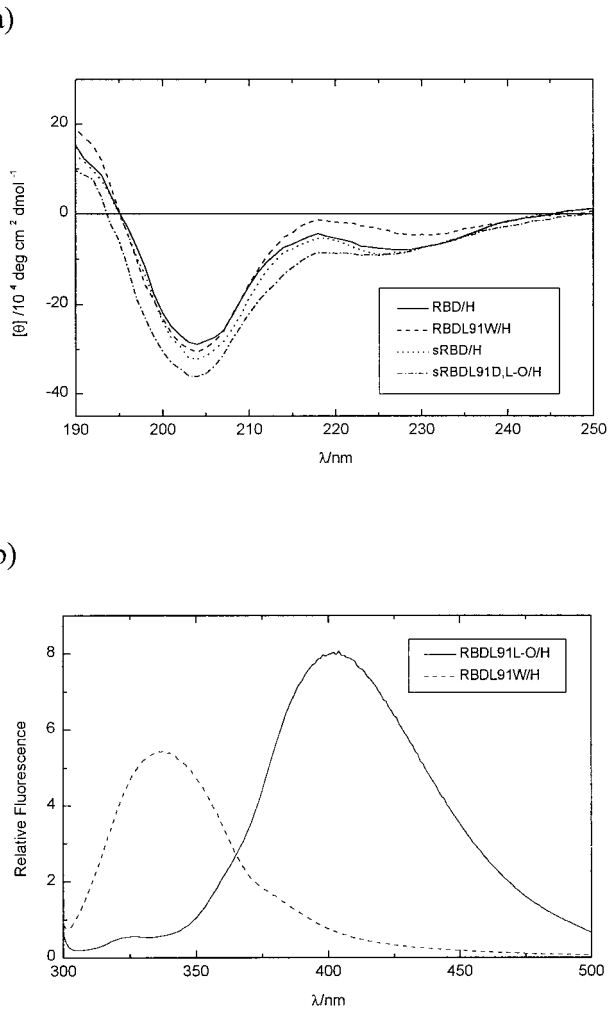

FIG. 4. (*a*) Far-UV CD spectra of chemically synthesized proteins (csRBD/H, cs[L91D,L-O]RBD/H) and RBD proteins expressed in *E. coli* (RBD/H, [L91W]RBD/H). Spectra were obtained at 20°C in 20 mM phosphate, pH 7.4 with a protein concentration of 95 μM. (*b*) Fluorescence spectra of cs[L91L-O]RBD/H and biosynthetic [L91W]RBD/H were measured at 25°C in 100 mM NaCl, 50 mM Tris·HCl, 5 mM $MgCl_2$, pH 7.4 with a protein concentration of 2 μM. The excitation wavelength was 290 nm.

tional properties of proteins. In these instances one could clearly distinguish between the D- and L-enantiomer. It should be mentioned that in some experiments a slow second phase in bindings kinetics of [L91L-O]RBD/H and [L91D,L-O]RBD/H to Ras could be observed. This phase showed no concentration dependence and was also apparent in displacement reactions where the dissociation rate of the complex was determined. Furthermore, these kinetic parameters varied widely from preparation to preparation. An explanation could be that a small fraction of misfolded synthetic protein (which would depend on the sample preparation) could give rise to this behavior.

CONCLUSION

We have shown that chemical synthesis is a viable method for preparing proteins modified in a manner that would be difficult on a large scale using molecular biological techniques (e.g., incorporation of an unnatural L-amino acid or perhaps in the future a D-amino acid).

In the particular example described here, a tryptophan derivative with fluorescent properties significantly different from those of tryptophan itself was incorporated into the

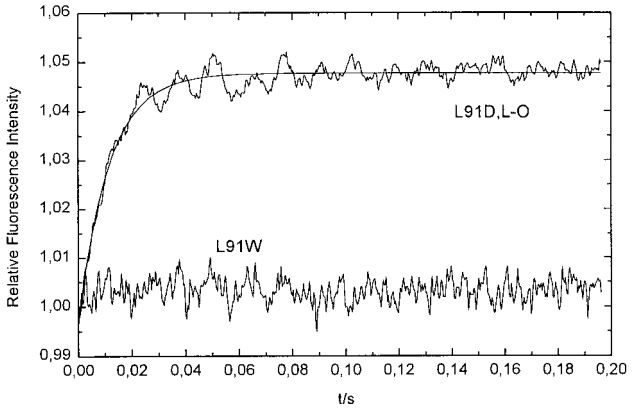

FIG. 5. Increasing fluorescence transient observed on rapid mixing of [L91D,L-O]RBD/H (0.5 μM) with H-Ras.mantGppNHp (2 μM) and transient observed for the rapid mixing of [L91W]RBD/H (0.5 μM) with H-Ras.mantGppNHp (2 μM) at 25°C under the same conditions. The excitation wavelength was 313 nm with detection through a 360-nm cutoff filter. The fitted curve of the increasing transient corresponds to a pseudo-first-order rate constant of 90 s^{-1}.

RBD. The chemically synthesized RBD protein interacted with Ras in a manner that was indistinguishable from that of biosynthetic wild-type RBD, and the fluorescence of the unnatural amino acid could be used to monitor this protein–protein interaction.

The work reported here is a prototype for the synthesis of small protein domains containing an artificial fluorophore for the study of protein–protein interactions. In future work, more sensitive labels that can be excited at wavelengths suitable for commercially available lasers should be sought, thus extending the range of applications of the modified proteins. Although the derivatives reported here are suitable for detailed mechanistic studies, more sensitive groups with longer wavelength excitation are potentially of great interest, for example in cell biological studies, for single molecule fluorescence measurements, and for sensitive screening techniques. These goals will be readily attainable for larger proteins with the recently reported expressed protein ligation method (37, 38).

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