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FATTY ACID ACYLATION OF RNase A USING REVERSED MICELLES AS MICROREACTORS

Stéphane Robert[a], Dominique Domurado[b], Daniel Thomas[a] and Joël Chopineau[a]

[a]Laboratoire de Technologie Enzymatique, URA CNRS 1442,

Université de Technologie de Compiègne, BP 649, 60206 COMPIEGNE,France

[b]Groupe de Pharmacocinétique des Conjugués Macromoléculaires (INSERM)

Centre de Recherche sur les Biopolymères Artificiels, URA CNRS 1465,

Faculté de Pharmacie, 34060 MONTPELLIER,France

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A water soluble protein, RNase A, was fatty-acylated using AOT reversed micelles in 2,2,4-trimethyl pentane as microreactors and myristoyl chloride as reagent. Artificial attachment of lipid molecules to this protein was performed for different hydration degrees by changing Wo= [water]/ [AOT], the parameter which controls the microreactor size. The chemically modified protein was monitored using reverse phase HPLC and characterized by HPLC, free amino groups titration, and electrophoresis. An RNase A/myristoyl chloride ratio of 1:4 (mol/mol) at Wo=7 was found to give 60 % of modified protein. © 1993 Academic Press, Inc.

Reversed micellar systems are composed of tiny aqueous droplets dispersed in an apolar medium, and stabilized by a monolayer of surfactant. Such amphiphilic molecules, comprising a hydrophilic head and a hydrophobic tail composed of one or two aliphatic chains, reduce the interfacial tension between both liquids, thus leading to a thermodynamically stable system. Reversed micellar media are isotropic and optically transparent.

Since reversed micelles compose a finely divided aqueous medium, they provide a useful tool to circumvent fundamental problems of protein chemistry difficult to approach in bulk water systems. It is now well established that enzymes and other biomolecules may be incorporated into the aqueous core of reversed micelles [1-3].

One of the great interests of these supramolecular structures consists in the possibility to adjust their diameter within a 10 nm (10^{-8} m) precision because the hydration degree, Wo= [water]/ [surfactant], controls the microreactor size. For instance, the refolding yield of denatured proteins has been improved by using reversed micelles to isolate denatured protein molecules from each other, and thus to reduce, upon refolding, the intermolecular interactions, which often lead to aggregation in standard aqueous solutions [4,5]. Several oligomeric enzymes have also been reported to dissociate into monomers or dimers during the entrapment into reversed micelles, permitting individual subunits studies [6-8].

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The protein molecules artificially hydrophobized by the introduction of a small number of aliphatic chains are assumed to acquire novel properties comparable to those exhibited by the *in vivo* acylated proteins [9-12]. Carrying out hydrophobization in water leads to a very substituted, then insoluble, product. Due to the fact that reversed micelles provide an aqueous microenvironment in a bulk organic phase, micellar media provide a means to "solubilize" proteins in apolar solvents, hence giving a possibility to modify them with water-insoluble reagents and allowing a controlled hydrophobization at a limited number of sites. No data published as yet enabling such a faithful protein modification, we performed a detailed study of RNase A myristoylation in the micellar system Aerosol OT/ 2,2,4-trimethyl pentane/ borate buffer.

RNase A is formed of a single peptidic chain composed of 124 amino acid residues. Its overall molecular mass calculated from its amino acid composition is 13680 Da. This water-soluble enzyme contains 11 lysine residues and exhibits a low surface hydrophobicity [13]. Its catalytic activity [14] and renaturing capacity [4,5] were studied in AOT reversed micelles. Finally, it can be obtained commercially in a very pure form.

Artificial attachment of lipid molecules to this protein was studied for different hydration degrees by changing Wo= [water]/ [AOT]. The pH and the ionic strength of the buffer used to solubilize RNase A are of great importance in this process. The chemical modification of the protein was monitored using reversed phase HPLC as an analytical method. The modified protein has been characterized through HPLC, free amino groups titration, and electrophoresis.

MATERIALS AND METHODS

Materials

Ribonuclease A (E.C. 3.1.27.5) from bovine pancreas was obtained from Boehringer Mannheim. Saturated fatty acid chlorides (capryl, myristoyl, palmitoyl and stearoyl) and Aerosol OT (used as received) were from Sigma. 2,2,4-trimethyl pentane, used after drying on 3 Å molecular sieves, came from Aldrich. Aceton was provided by Carlo Erba. Trifluoroacetic acid was from Fluka and acetonitrile (HPLC grade) from Riedel-de-Haën. Deionized water was filtrated through a MilliQ apparatus (Millipore).

RNase modification

RNase A was dispersed in 2,2,4-trimethyl pentane by incorporating it into reversed micelles of AOT using the injection method [3]. The experimental procedure was as follows: different volumes of the protein solution at different molarities in borate buffer (0.05 M, pH 7.8 - 10.1) were mixed in 25 ml of 0.1 M solution of AOT in 2,2,4-trimethyl pentane. Then 1 ml of saturated fatty acid chloride solution in 2,2,4-trimethyl pentane was added. Reagent concentrations were selected to give a defined molar ratio to the solubilized protein. The solution obtained was shaken during two hours on a rocking-stirrer at room temperature. In order to improve the overall protein yield, all manipulations were carried out in the same 50 ml polytetrafluoroethylen centrifugation tubes. The protein was isolated from the reaction mixture by precipitation upon addition of 25 ml of cold (-20°C) aceton. The protein precipitate was collected after a 20 min centrifugation at 2000 x g and -20°C. Then the following operation was repeated five times: the protein precipitate was washed with 10 ml of cold (-20°C) aceton; after 10 min of centrifugation at 2000 x g and -20°C, the supernatant containing 2,2,4-trimethyl pentane and AOT was discarded. Presence of AOT in the different fractions was checked by thin layer chromatography. Residual aceton was eliminated at room temperature by rotoevaporation.

HPLC analysis

The modified protein was analysed by a reverse-phase HPLC method. A Si C18 Nucleosil column (internal diameter 4.6 mm, 25 cm length, particles size 10 µm and 300 Å porosity) was used at 1 ml/min flow rate. A linear elution gradient was realized as follows: 0-2

min 100% A ; 2-20 min 0% to 95% B, with A: 0.05% of trifluoroacetic acid in water and B: 0.05% of trifluoroacetic acid in acetonitrile. The column temperature was maintained at 30°C. In order to identify the modified protein, two standards were used, the native RNase A and the control RNase A having undergone a modification procedure without the addition of reagent. The spectroscopic measurements were performed at 280 nm. The putative protein peaks were collected separetely and spotted on electrophoresis gels.

Electrophoresis

SDS polyacrylamide gel electrophoresis of native, control, unmodified and modified RNase A samples were performed on a high density gel using a PhastSystem™ (Pharmacia). Molecular weight markers used were in the range 2512 to 16949 daltons.

Free amino groups titration

The overall samples of modified RNase A were analysed using the method described by Fields [15]: a 0.5 ml sample (containing 5 to 200 nmols free amino groups) was mixed with 0.5 ml borate buffer (0.1 M, pH 9.5). Then, 33 µl of 1.8 M trinitrobenzene sulfonic acid were added, and the solution obtained was shaken for 5 minutes. Finally, 2 ml of a solution containing NaH_2PO_4, $NaSO_3$ (98.5 : 1.5), 0.1 M were added to stop the reaction. Optical density was measured at 420 nm.

RESULTS AND DISCUSSION

The process of protein acylation using reversed micelles as aqueous microreactors for solubilizing the enzyme, whereas the reagent is added into the organic phase consisted in three main steps:

- (i) incorporation of the protein into reversed micelles was carried out using the injection method. It has already been studied for RNase A [4,5,14].
- (ii) controlled chemical modification of the enzyme.
- (iii) recovery of the modified protein, as a water soluble fraction, from the reaction mixture.

Influence of the surfactant hydration degree on protein modification and recovery

The size of the inner cavity of reversed micelles can be widely varied by changing the surfactant:water ratio, Wo. Accordingly, protein modification degree and protein recovery have been studied for different values of this parameter.

As a control experiment, recovery of native RNase A from reversed micelles by acetone precipitation was performed for Wo values ranging between 6 and 25. For Wo=6, incorporation of this protein is possible as suggested by the limpidity of the mixture, but aceton precipitation did not result in the separation of the protein from the surfactant. For the other values, starting with 10 mg of protein, 70 to 85% of material were recovered without dependence on Wo.

RNase A acylation in AOT reversed micelles of different size was performed by varying Wo (Fig. 1). Protein modification degree and modified protein recovery decreased when Wo increases. The maximum recovery of protein modified albeit still water soluble was obtained for Wo=7. This surfactant hydration degree corresponds to a theoretical inner radius of empty reversed micelles of 14.5 Å [16] which is inferior to the 18 Å gyration radius of the protein [13]. Incorporation of RNase A into the aqueous droplets probably resulted in the formation of larger micelles as noticed previously [14]. Also, it is possible that the micelle size distributes somewhat about the mean. In this case, the protein could locate into the largest micelles. Nevertheless, both the degree of protein acylation and the recovery of modified protein were

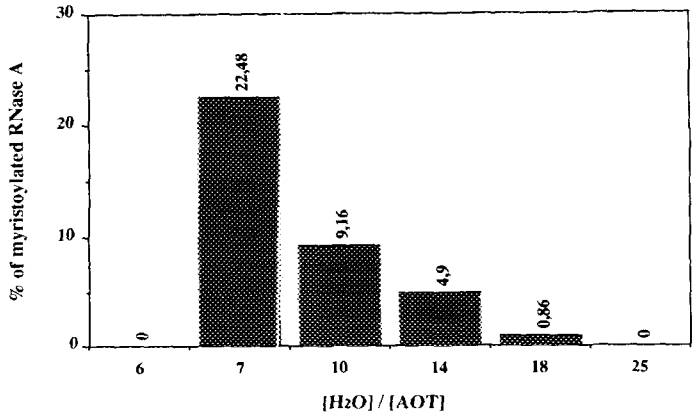

Fig. 1. Dependence of RNase A myristoylation on the hydration degree Wo in AOT reversed micelles in 2,2,4-trimethyl pentane. Protein:reagent=2:1, pH=10.1, 2.5mg of protein.

found to be optimum for the lowest Wo which allows to recover the native protein from AOT reversed micelles. As the micelles get smaller, the water layer around the protein gets thinner, and the distance between the surface of the protein and the polar medium, to be crossed by the acyl chloride head as a prerequisite for its reaction with the proteic amino groups, is smaller.

Effect of protein concentration and reagent:protein molar ratio on RNase A modification degree

Protein concentration is an important parameter for tailoring macromolecules in reversed micelles microreactors as described for RNase A refolding [4,5] or for triosephosphate isomerase reactivation [8]. The influence of protein quantity on this tailoring procedure was studied with a reagent:protein ratio of 2:1 and a Wo=7 (Fig. 2). The fraction of myristoylated protein increases with the protein amount from 0 to 12.5 mg, and then decreases. The water soluble fraction of the 10 mg sample is more homogeneous as evidenced by HPLC than the corresponding 12.5 mg sample. Consequently, 10 mg of protein was the amount chosen for further experiments.

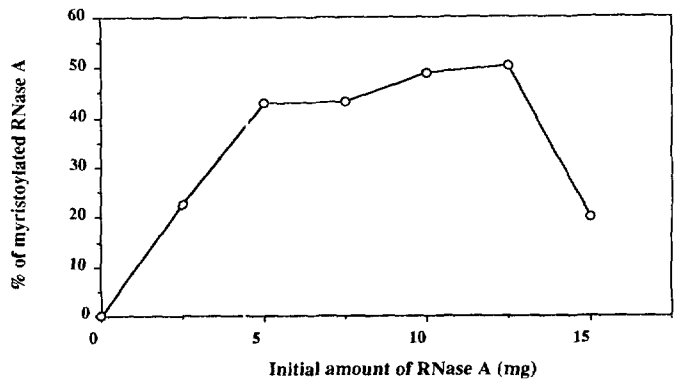

Fig. 2. Influence of protein concentration on the degree of RNase A myristoylation. Protein:reagent=2:1, Wo=7, pH=10.1.

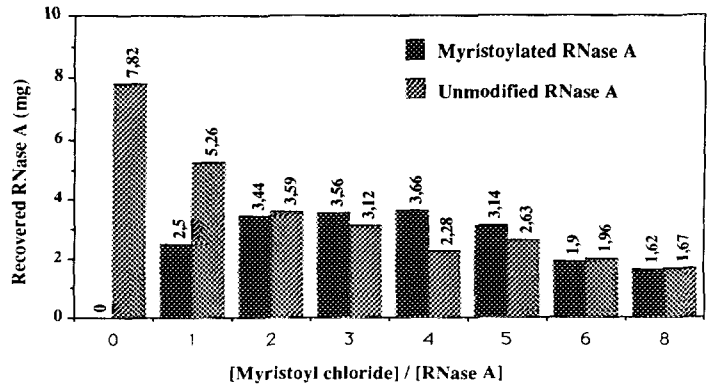

Fig. 3. Dependence of RNase A myristoylation on the protein:reagent ratio. Wo=7, pH=10.1, 10 mg of protein.

Protein modification was studied for different myristoyl chloride:RNase A ratios (Fig. 3). An optimal ratio of 4:1 was found to give more than 60 % of modified protein after recovery. Quantities of modified and unmodified protein after revovery are function of the reagent:protein ratio. As this molar ratio increases, the number of modified sites on the protein increases, thus leading to a decrease in the water soluble fraction. The correlation between modification degree and protein recovery results in a low overall recovery yield when the reagent ratio is superior to 4, especially when the degree of modification reaches the level where the modified protein hydrosolubility vanishes. Protein modification by acylating agents is usually performed using a large excess of reagent in order to get a high modification degree. For example, a 200-fold excess was used in the case of L-asparaginase acylation with palmitoyl chloride [17]. One advantage of the reverse micellar system is that varying the molar ratio from 1 to 8 gives the possibility to have a controlled modification degree of the protein.

Influence of aqueous core pH on the RNase hydrophobization

pH of the aqueous compartment, where the protein is located, is another parameter of importance. For instance, location and distribution of three proteins, chymotrypsin, cytochrome c and RNase A, have been studied in AOT reversed micelles [18]. One interesting point for our study was that interaction of protein in the reversed micelle with the surfactant layer is function of the pH of the water pool. Using 10 mg of RNase A at Wo = 7 and a myristoyl:protein reagent ratio of 4:1, the modification was assayed with the protein present in buffer of different pH. The results obtained are shown in Table 1.

Table 1 Influence of pH on the RNase A myristoylation.
Protein:reagent=4:1, Wo=7, 10 mg of protein.

pH	% acylated RNase A
7.8	0
9.3	9.4
9.5	41.7
10.1	61-64
12.4 (NaOH)	no RM formed

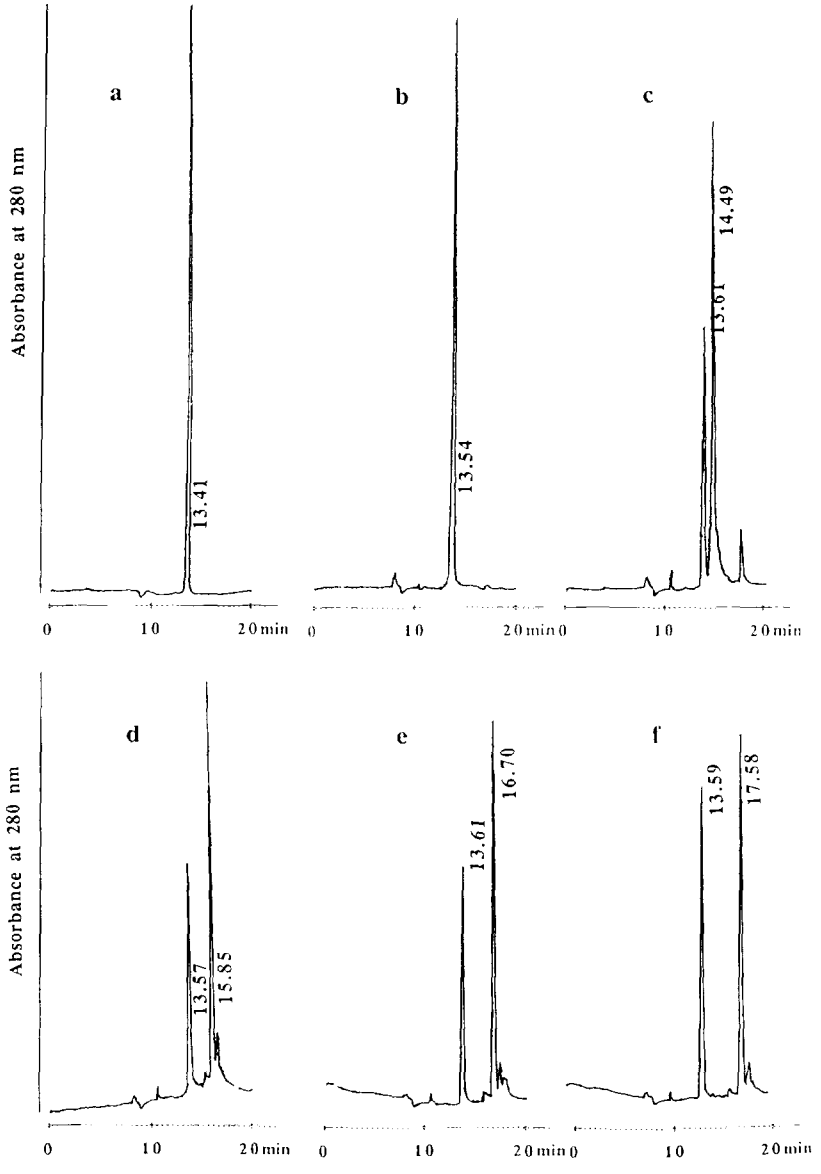

Fig. 4. Reversed phase HPLC profile of RNase A acylated with different fatty acid chlorides. a: Native, b: Control, c: C10, d: C14, e: C16, f: C18 modified RNase A.

The reaction was found to be pH-dependent. Starting with no modification at pH=7.8, a noticeable acylation took place when pH reaches 9.5 which corresponds to RNase A pHi [13]. The optimal conditions were obtained at pH=10.1. Explanation could be a combination of two events: protein location inside the micelle, moving from the aqueous core to the surfactant barrier as the pH increased and allowing a better access of the acyl chloride to the protein, and a change in exposure and reactivity of amino groups below and above RNase A pHi.

Table 2 Acylated amino groups after RNase A modification with different fatty acid chlorides. Protein:reagent=4:1, Wo=7, pH=10.1, 10 mg of protein.

Samples	Modified amino groups
Control RNase A	0
Caprylated RNase A	1.47
Myristoylated RNase A	1.12
Palmitoylated RNase A	1.97
Stearoylated RNase A	0.91

Acylation of RNase A with fatty acid chlorides of different chain length

Using the optimal procedure developped with myristoyl chloride, the protein was modified with capryl, myristoyl, palmitoyl and stearoyl chlorides. We obtained from 60 % for myristoyl, palmitoyl and stearoyl chlorides up to 68% of modified protein with capryl chloride. HPLC profiles of these four acylated proteins (Fig. 4) show an increased retention time as the aliphatic chain lengthens. Free amino group titration was carried out on native, control, caprylated, myristoylated, palmitoylated and stearoylated RNase A. While RNase A contains 11 lysine residues, only 7 amino groups are accessible to TNBS in this molecule [5]. The results, reported in Table 2, show that 1 to 2 aliphatic chains are linked to each enzyme molecule. The electrophoregram demonstrates the presence of protein in each of these 4 samples, the molecular mobility of which is not modified as compared to the native protein. All these facts support the idea of a controlled chemical modification.

From our experiments, we can conclude that the surfactant:water ratio, the protein concentration, the reagent:protein molar ratio and the pH of the aqueous compartment are pertinent parameters for the controlled hydrophobization of RNase A with fatty acid chlorides using reversed micelles of AOT in 2,2,4-trimethyl pentane. The optimal conditions were as follows: pH=10.1, Wo=7, protein:myristoyl chloride=4:1, 10 mg of protein in 25 ml of 0.1 M AOT in 2,2,4-trimethyl pentane. They were applied for acylation with other fatty acid chlorides. The aliphatic chain length does not influence crucially the result.

We defined optimal experimental conditions for RNase A. They are likely to have to be adapted for other proteins.

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REFERENCES

1. Martinek, K., Levashov, A.V., Klyachko, N.L., Khmelnitsky, Yu. L. and Berezin, I.V. (1986) Eur. J. Biochem. 155, 453-468.
2. Luisi, P. L. (1985) Angew. Chem. Int. Ed. Engl. 24, 439-450.
3. Luisi, P. L., Giomini, M., Pileni, M.P. and Robinson, B. H. (1988). Biochim. Biophys. Acta. 947, 209-246.
4. Hagen, A. J., Hatton, T. A. and Wang, D. I. C. (1990) Biotech. Bioeng. 35, 955-965.
5. Hagen, A. J., Hatton, T. A. and Wang, D. I. C. (1990) Biotech. Bioeng. 35, 966-975.

6. Kabanov, A. V., Nametkin, S. N., Evtushenko, G. N., Chernov, N. N., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) Biochim. Biophys. Acta.996,147-152.
7. Kabakov, V. E., Merker, S., Klyachko, N.L., Martinek, K. and Levashov, A.V. (1992) FEBS lett. 311, 209-212.
8. Garza-Ramos, G., Tuena de Gomez-Puyou, M., Gomez-Puyou, A. and Gracy, R.W. (1992) Eur. J. Biochem. 208, 389-395.
9. Kabanov, A. V., Levashov, A.V. and Martinek, K. (1987) Ann. N. Y. Acad. Sci., 501, 63-66.
10. Levashov, A.V., Kabanov, A. V., Khmelnitsky, Yu. L., Berezin, I.V. and Martinek, K. (1984) Dolk. Akad; Nauk SSSR. 278, 246-248 (in Russian) ; Dan (Engl. ed.) (1985) 278,295-297.
11. Kabanov, A. V., Levashov, A.V. and Alakhov, V. Y. (1989) Protein Eng. 3, 39-42.
12. Torchilin, V. P., Omelyanenko, V.G., Klibanov, A.L., Mikhailov, A. I., Goldanski, V. I. and Smirnov, V. N. (1980) Biochim. Biophys. Acta. 602, 511-521.
13. Richards, F. M. and Wyckoff, H. W. (1971) in The Enzymes Vol. 4 pp 647-784. P.A. Boyer 3rd ed. Academic Press.
14. Wolf, R. and Luisi, P. L. (1979) Biochem. Biophys. Res. Comm. 89, 209-217.
15. Fields, R. (1971) Biochem. J. 124, 581-590.
16. Klyachko, N.L., Levashov, A.V., Kabanov, A. V., Khmelnitsky, Yu. L. and Martinek, K. (1991) in: Kinetics and Catalysis in Microheterogeneous Systems (Gratzel, K. and Kalyanasundaram, K. eds) pp135-181, Marcel Dekker, New-York.
17. Martins, M. B., Jorge, J. S. and Cruz, M. E. (1990) Biochimie. 72, 671-675.
18. Petit, C., Brochette, P. and Pileni, M.P. (1986). J. Phys. Chem. 90, 6517-6521.