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1-N^6-Etheno-ADP-ribosylation of elongation factor-2 by diphtheria toxin

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Diphtheria toxin fragment A is able to inhibit protein synthesis in the eukaryotic cell by ADP-ribosylating the diphthamide residue of elongation factor-2 (EF-2) [(1980) J. Biol. Chem. 255, 10710–10720]. The reaction requires NAD as ADP-ribose donor. This work reports on the capacity of an NAD analog, the nicotinamide 1-N^6-ethenoadenine dinucleotide (εNAD), to be a substrate of diphtheria toxin fragment A in the transferring reaction of the fluorescent moiety, the εADP-ribose, to the EF-2. As a consequence of the transfer of the εADP-ribosyl moiety to the EF-2, there is an increase in the emission intensity of the fluorophore and a blue shift in its emission maximum. The εADP-ribosylated EF-2, like ADP-ribosylated EF-2, retains the capacity to bind GTP and ribosome. The utility of introducing a fluorescent probe in a well defined point of the EF-2 molecule for conformational or binding studies is discussed.

Elongation factor-2 *Diphtheria toxin* *εNAD* *Etheno-NAD* *ADP-ribosylation*

1. INTRODUCTION

The nicotinamide 1-N^6-ethenoadenine dinucleotide (ϵNAD) is a fluorescent analog of NAD which has been shown to be interchangeable with NAD in the binding with several dehydrogenases [2,3]. It exists in a stacked conformation in solution but assumes an open form, which has a much higher quantum yield, when bound to the dehydrogenases [2–4]. Its fluorescence maximum is around 410 nm and can be excited above 305 nm, a region where the aromatic chromophores of proteins do not interfere [2–4].

Diphtheria toxin fragment A transfers the ADP-ribose moiety from NAD to an acceptor site on the EF-2 molecule, which has been identified as a post-translationally modified histidine residue, called diphthamide [1]. The ADP-ribosylated EF-2 no longer catalyzes the translocation, although it still retains the capacity to bind GTP and to form a ternary complex with ribosomes [5].

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Here, we show that diphtheria toxin fragment A is able to transfer the ϵADP-ribose moiety from ϵNAD to the EF-2 molecule. Some fluorescence properties of ϵADP-ribosylated EF-2 are reported. Moreover, we examine the behaviour of ϵADP-ribosylated EF-2 in GTP and ribosome binding.

2. MATERIALS AND METHODS

2.1. *Purification of ϵNAD*

ϵNAD was purchased from P-L Biochemicals and purified by high-performance liquid chromatography (HPLC), using an Altex Ultrasil-Ax column equilibrated with 50 mM formic acid and eluted with the same solvent. Concentrations of ϵNAD were determined at pH 7 using a molar extinction coefficient of 1.0×10^3 at 265 nm [3].

2.2. *Purification of EF-2 and preparation of ϵADP-ribosylated EF-2*

EF-2 was obtained from human placenta by an original procedure (in preparation) which, briefly, consists in precipitation at pH 5 of the

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postmitochondrial fraction and sequential chromatographies on DEAE-cellulose, heparin-Sepharose and Sephadex G-150 resins. To obtain the ϵADP-ribosylated protein, EF-2 (1 mg/ml) was incubated for 60 min at 25°C in the presence of fragment A (1 μg/ml), purified as in [6], with 50 μM ϵNAD in a buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA and 5 mM dithiothreitol.

2.3. *Reversal of ADP-ribosylation*

The ϵADP-ribosylated EF-2 (0.5 mg/ml) was incubated at pH 6.5 in the presence of fragment A (10 μg/ml) and nicotinamide (2 mM) at 25°C. The reaction products were analyzed by HPLC as in section 2.1 after deproteinization with formic acid.

2.4. *Digestion of ϵADP-ribosylated EF-2 with phosphodiesterase*

The ϵADP-ribosylated EF-2, obtained as described in section 2.2, was exhaustively dialyzed against the incubation buffer and then chromatographed on a Sephadex G-25 column, to eliminate completely the ϵNAD excess. Phosphodiesterase from *Crotalus durissus* (Boehringer, Mannheim) was then added to the protein solution to a final concentration of 10 μg/ml. The solution was allowed to stand for 30 min at 37°C. The reaction products were analyzed as described in section 2.1 after deproteinization with formic acid.

2.5. *Fluorescence measurements*

Fluorescence spectra and intensities were obtained with a Perkin-Elmer MPF-44B spectrofluorometer. Fluorescence measurements were made in the range where emission was linear with ϵNAD concentration. The absorbance of all solutions was less than 0.1 at the excitation wavelength. The temperature of the solution cells was maintained at 25°C.

3. RESULTS

Incubation of EF-2 with ϵNAD in the presence of diphtheria toxin fragment A results in a time-dependent enhancement of the fluorescence emission at 410 nm with a blue shift of the emission maximum of about 5 nm. Fig.1 (inset) reports the time course of this reaction under the conditions specified in the legend, while curves a and b represent the emission spectra of the incubation mixture, excited at 320 nm, before the addition of fragment A and at the end of the reaction.

It is known that upon binding to proteins, such as some dehydrogenases, the fluorescence of ϵNAD increases due to the separation of the nicotinamide and ethenoadenine rings which are partially stacked in the free molecule [2–4]. Actually, so far, no reaction in which a moiety of the ϵNAD is transferred has been reported. The following experiments demonstrate that, in the case of the reaction of EF-2 with ϵNAD, the fluorescence increase has to be ascribed to the transfer of the ADP-ribose moiety to the protein. In fact, the incubation of ϵNAD in the presence of EF-2, without fragment A, does not result in a fluorescence increase. Thereafter we can exclude that, if binding occurs, it leads to a quantum yield variation of ϵNAD (as in the case of its binding to

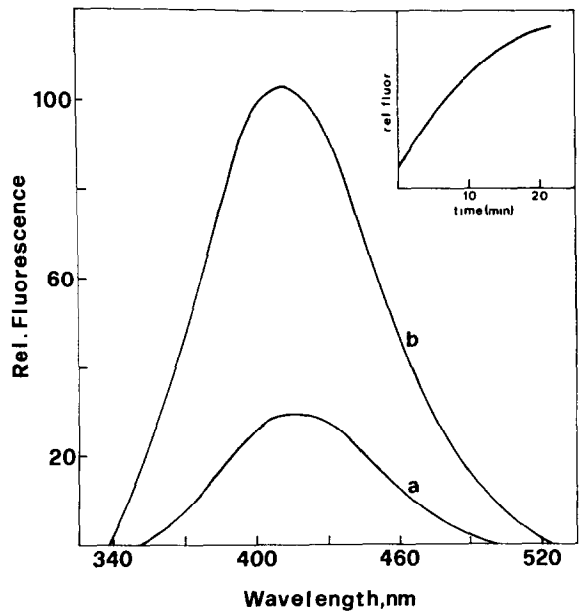

Fig.1. Fluorescence emission spectra of the incubation mixture before the addition of fragment A (curve a) and at the end of the reaction (curve b). Excitation was at 320 nm. The incubation mixture contained: EF-2 (1 mg/ml), ϵNAD (5 μM), fragment A (1 μg/ml). Inset: time course of the reaction monitored by the fluorescence emission increase at 410 nm.

the dehydrogenase). Thus the observed fluorescence increase, after the addition of fragment A, is more likely due to the unstacking of the adenine ring from the nicotinamide ring as a consequence of the transfer of the ϵADP-ribose moiety to EF-2.

Moreover, after exhaustive dialysis of the incubation mixture (also containing fragment A), against either the incubation buffer or incubation buffer plus 6 M guanidine hydrochloride, the fluorescence of the dialyzed mixture was retained.

Another result consistent with the hypothesis of a fluorophore covalently bound to the EF-2 was also obtained from chromatography of the incubation mixture on a Sephadex G-25 column equilibrated in 6 M guanidine hydrochloride. Two fluorescent peaks emerged from the column: the first, containing the protein, in the excluded volume, the second, containing the free ϵNAD, in the void volume region.

Treatment of the incubation mixture with phosphodiesterase and successive dialysis against the incubation buffer led to the complete loss of the fluorescence of the dialyzed mixture. The phosphodiesterase is able to cleave the 5′-AMP from the ADP-ribosylated EF-2 [7]. The incubation mixture was loaded on a Sephadex G-25 column equilibrated with incubation buffer. The front fluorescent peak was treated with phosphodiesterase: a substance was produced which had the same retention peak in HPLC as

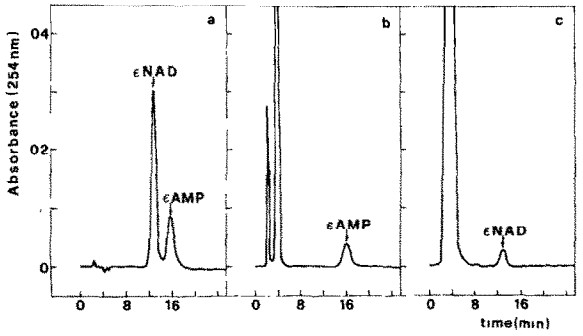

Fig.2. HPLC elution pattern of: a standard mixture of ϵNAD and 5′-ϵAMP (a); phosphodiesterase digestion products of ϵADP-ribosylated EF-2 (b); products from the reversal of ϵADP-ribosylation by fragment A and nicotinamide (c). Column, Altex Ultrasil Ax, 0.46–25 cm; eluent, 50 mM formic acid; flow rate, 1 ml/min.

5′-ϵAMP (fig.2b). On the other hand, incubation of the front fluorescent peak with fragment A and adenosine[U-^{14}C]NAD does not result in incorporation of radioactivity in the trichloroacetic acid-precipitable fraction.

It is well known that fragment A is able to reverse the ADP-ribosylation of EF-2 in the presence of nicotinamide, forming NAD and EF-2 [8]. The pH optimum for the reverse reaction is lower than that of the forward reaction. Incubation of the front fluorescent peak from Sephadex G-25 with fragment A and nicotinamide at pH 6.5 produces a substance which has the same chromatographic behaviour as ϵNAD in HPLC (fig.2c).

4. DISCUSSION

The ADP-ribosylation reaction catalyzed by fragment A is highly specific for eukaryotic EF-2. Moreover, it has been proved that EF-2 has a unique attachment site for ADP-ribose, i.e. diphthamide, an amino acid resulting from the posttranslational modification of a histidine residue [1]. Diphthamide has been found in all examined eukaryotic cells [9]. As a consequence of ADP-ribosylation, EF-2 becomes unable to catalyze the translocation although it retains the capacity to bind GTP and ribosome in forming the ternary complex [5]. Therefore, the possibility of specifically binding a fluorescent probe, having suitable spectral features, to the diphthamide could be of great utility in studying, with the power of fluorescence techniques, the molecular district of EF-2 containing that crucial residue. The 1-N^6-etheno-ADP-ribosyl moiety does have those features. It has, first of all, a high structural similarity to the ADP-ribosyl moiety and possesses, among spectral aspects, high quantum yield, high intrinsic polarization, long lifetime, and excitation and emission spectra clearly resolved from those of the protein fluorophores [10]. Moreover, ϵADP-ribosyl labelling of diphthamide is achieved with an efficient and selective enzymatic mechanism which assures, besides the specificity, the uniqueness of the labelled site on the protein. The specificity also allows the use of a poorly purified EF-2.

Our results leave no doubt that ϵNAD is interchangeable with NAD as a substrate of fragment A

in the ADP-ribosylation of EF-2 and in the reverse reaction. At this point the question arises of whether ϵADP-ribosylated EF-2 retains the same molecular properties as ADP-ribosylated EF-2. So far, we have found that ϵADP-ribosylated EF-2 behaves like ADP-ribosylated EF-2 in forming the ternary complex with GTP and ribosome. In fact, after incubation with ribosomes and GTP, a fluorescence, typical of ϵADP-ribosylated EF-2, is observed in the resuspended ribosome pellet. Experiments aiming to clarify the structural aspects of the complex of the ϵADP-ribosylated EF-2 with GTP and ribosome are presently in progress.

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