Volume 278, number 2, 225–228 FEBS 09363 January 1991

ADONIS 0014579391000748

Examination of elongation factor Tu for aluminum fluoride binding sites using fluorescence and ^{19}F-NMR methodologies

Theodore L. Hazlett[1], Tsutomu Higashijima[2] and David M. Jameson[1]

[1]*Department of Biochemistry and Biophysics, University of Hawaii at Manoa, 1960 East-West Road, Honolulu, Hawaii 96822* and [2]*Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235, USA*

Received 25 September 1990, revised version received 15 November 1990

This article reports on a comparison of the interaction of Al^{3+} and F^- with two GTP-binding proteins, elongation factor Tu (EF-Tu) and the hormone sensitive regulatory protein (G protein) $G_o\alpha$. The methodologies chosen to elucidate possible interactions between protein and aluminum fluoride were fluorescence spectroscopy and nuclear magnetic resonance (^{19}F-NMR). Both proteins have tryptophan residues near their nucleotide binding sites, the purported site of aluminum fluoride interaction. It has been assumed for G proteins (including $G_o\alpha$) that aluminum fluoride, in the presence of Mg^{2+}, mimics the magnesium coordinated γ-phosphate group for the GDP-form of the protein and shifts the protein's conformation toward the active GTP-form. Indeed, changes in intrinsic fluorescence of $G_o\alpha$ effected by aluminum fluoride are observed. The presence of aluminum fluoride did not affect the intrinsic fluorescence, spectra or lifetimes, of EF-Tu·GDP. ^{19}F-NMR was then used to directly test for bound F^-. Fluoride alone or in the presence of either protein gave a single ^{19}F-NMR peak at −10 ppm, characteristic of free F^-. With the addition of aluminum to the protein and F^- samples a second peak, shifted upfield from the first to −29 ppm, was observed for $G_o\alpha$·GDP. This second peak, which has been assigned to protein-bound F^-, was not observed for EF-Tu·GDP. These observations show that the interaction of Al^{3+} and F^-, in the presence of Mg^{2+}, may be quite different between the hormone-sensitive G proteins, which bind aluminum fluoride, and the GTP-binding proteins as a whole, which include EF-Tu. Care must therefore be exercised when structural data on the elongation factor, specifically on the nucleotide site, are used to interpret data or compose models intended to describe the hormone-sensitive regulatory G proteins.

Elongation Factor Tu, Aluminum Fluoride, $G_o\alpha$, F-NMR, Fluorescence

1. INTRODUCTION

Interest in aluminum fluoride stems from early reports demonstrating that a variety of enzymes are influenced by the presence of fluoride ions. Kinases and phosphatases are inhibited [1] while adenylyl cyclases are generally activated [2]. Later it was discovered, in the case of adenylate cyclase activation, that fluoride interacted with G_s, a GTP-binding regulatory protein (G protein) which couples the membrane receptor to adenylyl cyclase. In 1982 Sternweis and Gilman demonstrated that the presence of trace amounts of aluminum were required to activate adenylyl cyclase with fluoride [3]. The working hypothesis was then expounded by Bigay et al. [4,5] that aluminum fluoride may be a γ-phosphate analogue binding to the guanine nucleotide site at the γ-phosphate position, vacant in the presence of GDP. Bound, the aluminium fluoride would then confer a GTP-like conformation on the G protein. At present, all regulatory G proteins are known to interact with Al^{3+} and F^-, in the presence of Mg^{2+}, and, indeed, the influence of fluoride ions on a particular event is often sited as evidence for G protein interaction.

Here we have applied ^{19}F-NMR to directly measure bound and free fluoride and have applied steady-state as well as time-resolved fluorescence techniques to monitor changes in the local protein conformation as a consequence of aluminum fluoride binding. Both methods lead to complementary results toward evaluating possible interactions between the proteins and the aluminum fluoride complex.

2. MATERIALS AND METHODS

Guanosine 5′-diphosphate (GDP), phenylmethanesulfonyl fluoride (PMSF), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Biochemicals. [8-^{3}H]GDP was obtained through Amersham, and dithiothreitol (DTT) was from Pierce Chemical Co. $AlCl_3$ was from Mallinkrodt. $MgCl_2$ and NaF were purchased from J. T. Baker Chemical Co.

EF-Tu was purified from *Escherichia coli* MRE600 paste (Grain Processing, Inc.) by the method of Leberman et al. [6]. GDP-binding activity of the EF-Tu was determined using the filter assay as described by Miller and Weissbach [7], and the total EF-Tu·GDP concentration was determined using an extinction coefficient at 280 nm of 29 200 M^{-1} cm^{-1} [8]. Preparations were routinely stored at −80°C in 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM DTT, 10 μM GDP, and 10 μM PMSF. All measurements were performed at 20°C. The EF-Tu NMR samples were put into buffer containing 80% D_2O.

Correspondence address D.M. Jameson, Department of Biochemistry and Biophysics, University of Hawaii at Manoa, 1960 East-West Road, Honolulu, Hawaii 96822, USA

Bovine brain $G_{o\alpha\gamma}$ was purified as reported previously [9,10] in the presence of aluminum fluoride. Aluminum fluoride-free $G_{o\alpha\gamma}$ was prepared, as reported, by consecutive chromatography with hydroxylapatite and Q-25 $G_{o\alpha\gamma}$ was stored in 50 mM HEPES (pH 8), 1 mM EDTA, 1 mM DTT, 0.1% Lubrol and kept at -80°C until needed [11].

Technical fluorescence spectra were taken at 20°C on either a SPEX Fluorolog fluorometer or an SLM 8000C spectrofluorometer. The excitation wavelength was set to 300 nm, 4 nm band-pass, to avoid excitation of tyrosine residues for EF-Tu, and set to 290 nm, 4 nm band-pass, for $G_{o\alpha\gamma}$. In all cases a buffer blank was subtracted from the sample spectra though these values were less than 2% of the total sample intensity for EF-Tu and less than 10% for $G_{o\alpha\gamma}$.

The time-resolved fluorescence measurements were performed using time correlated single photon counting with laser/microchannel plate instrumentation described in detail elsewhere [12]. The excitation wavelength was set to 300 nm and the emission was monitored at 340 nm with a bandpass of 4 nm. The channel width was 20.5 ps and the data were collected in 1024 channels. In each case a sample blank was counted for the same accumulation time and subtracted from the sample decay curve.

^{19}F-NMR experiments were performed on a Varian VXR-500 (^{19}F resonance frequency is 470.27 MHz) at 20°C. Chemical shifts were measured relative to 10% trifluoroacetic acid in D_2O under the same experimental conditions as the EF-Tu·GDP protein sample. An average of 256 FID's (free induction of decays) each after a 40° pulse were accumulated at an interval of 3.5 s.

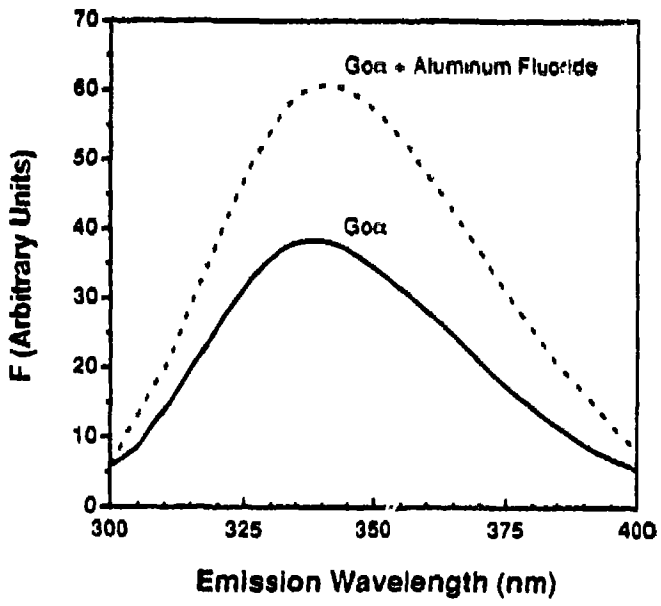

Fig. 1. The intrinsic fluorescence spectrum of $G_{o\alpha\gamma}$ (300 nm) is recorded with (——) and without (----) Al^{3+} (20 μM), F^- (10 mM), and Mg^{2+} (6 mM). Excitation wavelength was 290 nm and the spectra were recorded at 20°C.

3. RESULTS AND DISCUSSION

G proteins as a group show much homology in their physiological characteristics (for reviews see ref. 13 and 14). The underlying common feature among the G proteins relevant to this work is the conformational difference between the GTP and GDP forms. The GTP form has an increased affinity for the respective effector while the GDP form binds to the receptor. The exact nature of the resulting conformational change is not presently known for any of the G proteins under study. Of the GTP-binding proteins being investigated, EF-Tu is probably the best understood with data on the crystal structure for the GDP form having been reported [20,21]. Other G proteins, with the exception of the ras p21 proteins [15–17], have yet to be crystallized and little is known about their overall structure.

The fluorescence results on $G_{o\alpha}$ [9] demonstrate a 60% increase in the protein's intrinsic fluorescence upon binding of aluminum fluoride (Fig. 1). Hence one or more of the $G_{o\alpha}$ tryptophans is markedly influenced by the presence of aluminum fluoride, a result consistent with the idea that aluminum fluoride binds to the protein with a resultant conformational change. From previous work it is evident that 1 mol of aluminum binds per mole of protein with the site in the γ-phosphate local of the nucleotide binding domain ([4,5] and Higashijima et al., in preparation). Since the presence of the salt complex has been shown to reversibly modify the behavior of several G proteins toward their effectors, one can assume that the association is not denaturing. Indeed, the association appears to activate the G protein. This conclusion is also supported by the further observation that the binding of GTPγS, a non-hydrolyzable GTP analogue, to $G_{o\alpha}$ induces similar fluorescence changes as well as activates the protein [18].

Fluorescence enhancement of the EF-Tu tryptophan has been observed in the cases of EF-Tu denaturation in 6 M guanine HCl (Hazlett and Jameson, unpublished) or SDS [19] and for the GTP-form of the protein [19]. Information from the crystal structure [20,21] and sequence data [22] have assigned the single tryptophan to residue 184 within the GDP-binding domain and near the nucleotide site. Fluorescence quenching data have described the tryptophan environment as relatively inaccessible to the solvent [23] suggesting an interior facing orientation. This interpretation is reasonable since the tryptophan emission in EF-Tu·GDP [19,23] is highly quenched, a state which is often due to interaction with nearby amino acid residues. Thus, one would expect the tryptophan to be ideally situated to monitor aluminum binding to the nucleotide site through the resulting changes in the relative position of tryptophan with nearby residues and the solvent. However, in the presence of aluminum and fluoride the tryptophan fluorescence of EF-Tu·GDP showed little change (Fig. 2). The 3% decrease is most likely due to a small amount of denatured EF-Tu·GDP, presumably due to the temporary, local high ionic strength upon the addition of a small volume of concentrated aluminum chloride. A transient observable increase in the solution scattering immediately after $AlCl_3$ addition supports this contention.

Fluorescence lifetime data on EF-Tu, in the presence or absence of aluminum fluoride, were best fit to a

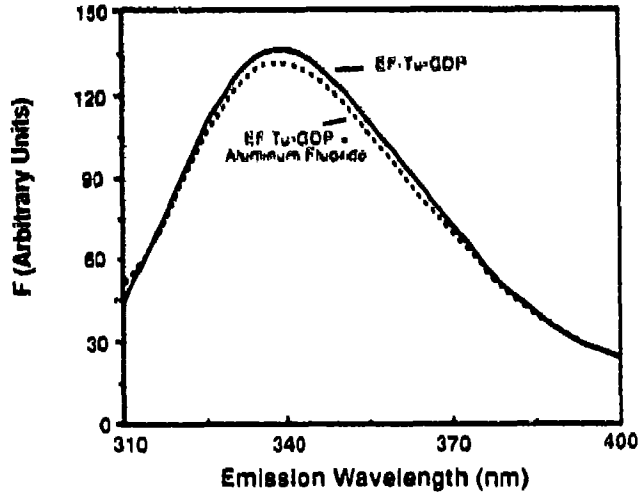

Fig. 2. Technical emission spectra of EF-Tu (2 μM), excitation at 300 nm, in the presence (- - -) and absence (——) of aluminum (100 μM) and fluoride (10 mM) are presented. The buffer also contained 5 mM Mg^{2+} and 10 μM GDP.

model of three exponential decays (with aluminum fluoride: 5.6 ns, 1.3 ns and 0.4 ns; without aluminum fluoride: 5.5 ns, 1.2 ns, and 0.37 ns) and a 0.0 ns delta function indicating a trace of a scattering component. The reduced χ^2, was 1.04 for both data sets, indicating a good fit. Interaction of EF-Tu·GDP and aluminum fluoride was not indicated.

Fluorine NMR results on both proteins reconfirmed our conclusions with $G_o\alpha$ (this report and Higashijima et al., in preparation) and further supported our conclusions for EF-Tu·GDP based on the fluorescence. Aluminum fluoride in solution gives a single peak at -10 ppm from trifluoroacetic acid, as does free fluoride, which remains unchanged in the presence of aluminum. Fluoride bound to the protein, presumably with aluminum and complexed with the appropriate protein side chains would be expected to have a shifted ^{19}F-NMR peak. This shift was clearly observed for $G_o\alpha$ in the presence of aluminum fluoride where peaks for both free fluoride, -10 ppm, and bound fluoride, -29 ppm, were observed (Fig. 3).

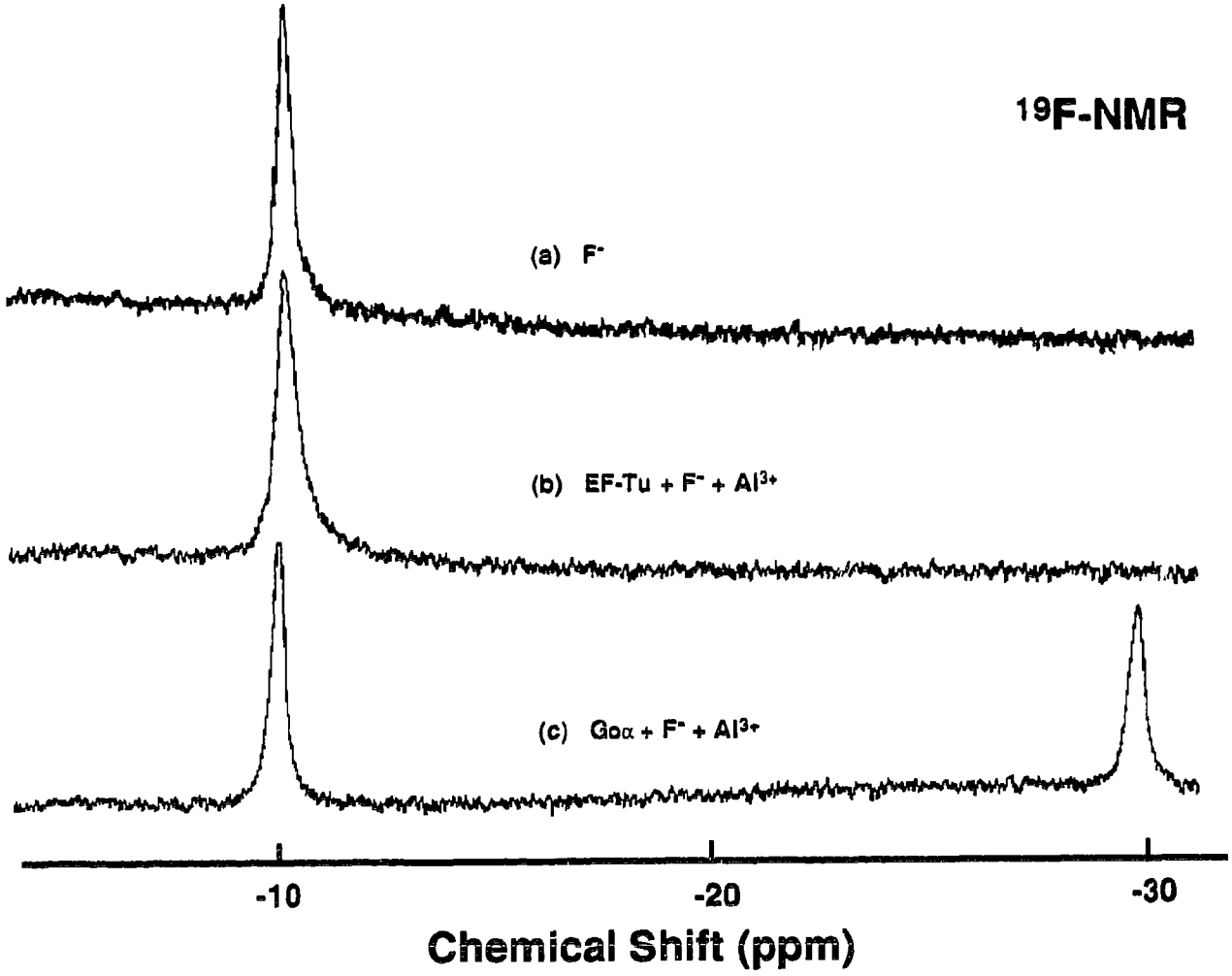

Fig 3 ^{19}F-NMR spectra in the presence of Mg^{2+} on Fluoride (a), EF-Tu (540 μM) in the presence of F^- (5 mM) and Al^{3+} (500 μM) (b), and $G_o\alpha$ (700 μM) in the presence of Al^{3+} (700 μM) and F^- (5 mM) (c)

Again in contrast to the $Q_{0\alpha}$ data, only the free fluoride peak at -10 ppm was observed for the EF-Tu·GDP sample. Additionally, a fast exchange between free F^- and protein-bound F^- is unlikely since there was no shift in the free F^- signal. The nucleotide site, then, appears to be sufficiently different from the $Q_{0\alpha}$ site to not recognize the aluminum fluoride as a γ-phosphate analogue. Our data and conclusion are in complete agreement with a recent report by Kraal and co-workers who, by examining the intrinsic GTPase activity and applying limited tryptic digestion methods, found no evidence of an aluminum fluoride induced GTP-form of the elongation factor [24]

This study and that of Kraal and co-workers [24] clearly demonstrate that aluminum fluoride does not act as a γ-phosphate analogue for EF-Tu·GDP. Consequently, the structural characteristics of the EF-Tu nucleotide site, despite the reported homologies among the GTP-binding proteins, must differ from the hormone-sensitive regulatory G proteins It should also be noted that ARF, another well-studied GTP-binding protein, does not appear to bind aluminum fluoride [25]. Just what these structural differences are and how the various GTP-binding proteins are related are questions which have yet to be answered. One must, therefore, take care in applying structural data across the family of GTP-binding proteins since differences in the physical structure of their nucleotide sites are in evidence

Acknowledgements We would like to thank Dr Lila Gierasch for the use of the Varian VXR-500 NMR apparatus and Dr Arthur Szabo for use of his lifetime equipment and his help with the EF-Tu intrinsic lifetime measurements This work was supported by the National Science Foundation Grants DMB-8706440 and DMB-9005195 (D M J) and by the United States Public Health Service Grant GM40676. D M J is an Established Investigator of the American Heart Association A preliminary report of this work has been presented at the Feb 1990 meeting of the Biophysical Society

REFERENCES

[1] Hewitt, E J and Nicholas, D J D (1963) in Metabolic Inhibitors (Hochster, R M and Quastrel, J H , eds) Academic Press, New York, 2, 31-421
[2] Rall, T.W. and Sutherland, E W (1958) J. Biol. Chem. 232, 1065-1076.
[3] Sternweis, P.C. and Gilman, A.G (1982) Proc Natl Acad. Sci. USA 79, 4888-4891.
[4] Bigay, J., Deterre, P., Pfister, C and Chabre, M. (1985) FEBS Lett 191, 181-185
[5] Bigay, J., Deterre, P., Pfister, C. and Chabre, M. (1987) EMBO J. 6, 2907-2913.
[6] Leberman, R , Antonsson, B., Giovanelli, R., Quarlguata, R , Shumann, R. and Wittinghofer, A. (1980) Anal Biochem 104, 29-36.
[7] Miller, D L. and Weissbach, H. (1974) Methods Enzymol. 30, 219-232.
[8] Hazlett, T.L., Johnson, A.E. and Jameson, D M. (1989) Biochemistry 28, 4109-4117.
[9] Higashijima, T , Ferguson, K.M., Sternweis, P.C., Rose, E.M., Smigel, M.D and Gilman, A G (1987) J. Biol. Chem. 262, 752-756.
[10] Sternweis, P.C. and Robishaw, J D. (1984) J. Biol. Chem 259, 13806-13813.
[11] Sternweis, P.C , Northrup, J.K., Smigel, M D and Gilman, A G. (1981) J Biol. Chem 256, 11517-11526
[12] Willis, K.J. and Szabo, A G. (1989) Biochemistry 28, 4902-4908.
[13] Allende, J.E (1988) FASEB J. 2, 2356-2367
[14] Gilman, A G (1987) Annu Rev Biochem 56, 615-649.
[15] DeVos, A.M., Tong, L , Milburn, M.V., Matias, P.M , Jancarik, J., Noguchi, S , Nishimura, S., Miura, K , Ohtsuka, E and Kim, S.-H (1987) Science 239, 888-893
[16] Jurnak, F (1988) Trends Biochem Sci. 13, 195-198.
[17] Tong, L , DeVos, A M., Milburn, M.V , Jancarik, J , Noguchi, S , Nishimura, S., Miura, K., Ohtsuda, E and Kim, S -H (1989) Nature 337, 90-93
[18] Higashijima, T , Ferguson, K M , Smigel, M.D and Gilman, A G (1987) J Biol Chem. 262 757-761.
[19] Arai, K , Arai, T , Kawakita, M. and Kaziro, Y (1977) J Biochem 81, 1335-1346
[20] Jurnak, F (1985) Science 230, 32-36
[21] LaCour, T.F M , Nyburg, J , Thirup, S and Clark, B F C (1985) EMBO J. 4, 2385-2388
[22] Arai, K , Clark, B F C., Duffy, L., Jones, M D , Kaziro, Y , Laursen, R A , L'Italien, J , Miller, D L , Nagarkelti, S , Nakamura, S , Nielsen, K M , Petersen, T.E , Takahashi, K and Wade, M (1980) Proc Natl Acad Sci USA 77, 1326-1330
[23] Jameson, D M , Gratton, E and Eccleston, J F (1987) Biochemistry 26, 3894-3901
[24] Kraal, B , de Graaf, J M , Mesters, J R , Van Hoof, P J M , Jacquet, E and Parmeggiani, A. (1990) Eur J Biochem 192, 305-309
[25] Kahn, R A and Gilman, A G (1986) J Biol Chem 261, 7906-7911