REDUCTIVE METHYLATION OF IF-3 AND EFTu WITH [^{14}C]FORMALDEHYDE AND SODIUM CYANOBOROHYDRIDE

Linda A. MacKEEN, Claudia DiPERI and Ira SCHWARTZ*

Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, USA

1. Introduction

The extent of interaction of a protein with a receptor is most easily determined when the protein is highly radioactive. It is often easiest to purify the nonradioactive protein and subsequently post-label by appropriate modification. The most commonly employed procedures are iodination of tyrosyl residues, which often leads to inactivation, and reductive alkylation with [^{14}C]formaldehyde and sodium borohydride as described [1].

We found, however, that reductive methylation of initiation factor-3 (IF-3) and elongation factor Tu(EFTu) with NaBH$_4$ at the required high pH yielded proteins of low specific radioactivity (3000–6000 cpm/μg). We therefore explored the use of a milder reductant, sodium cyanoborohydride, that could be employed at lower pH values. Reductive ^{14}C-methylation of IF-3 and EFTu with NaBH$_3$CN gave specific activities >60 000 cpm/μg protein. The advantages of this procedure and the effect of extensive methylation on the function of IF-3 and EFTu is presented.

2. Materials and methods

2.1. Materials

Sodium cyanoborohydride was purchased from Aldrich and [^{14}C]formaldehyde (42 mCi/mmol) was obtained from New England Nuclear. IF-3 was prepared according to [2] and EFTu · GDP according to [3].

* To whom all correspondence should be addressed

2.2. Reductive methylation of IF-3 and EFTu

Reductive methylation of IF-3 was performed in a buffer containing 10 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 5% glycerol and 800 mM KCl. Protein concentrations ranged from 1.0–1.8 mg/ml and methylations were successfully carried out with as little as 9.5 μg protein. Equimolar amounts of 0.25% [^{14}C]formaldehyde (42 mCi/mmol) and 1.0% NaBH$_3$CN were added at a 39-fold molar excess over IF-3 unless otherwise indicated. Mixtures were incubated at room temperature for 60 min, then extensively dialyzed against 5 changes of 250 ml 20 mM Tris (pH 7.6), 1 mM EDTA, 5% glycerol, 500 mM NH$_4$Cl.

The reaction with EFTu · GDP was carried out in 100 mM sodium borate (pH 9.0), 200 mM KCl, 10 mM MgCl$_2$, 20 μM GDP and 2 mM 2-mercaptoethanol. [^{14}C]Formaldehyde and NaBH$_3$CN were added as above, but at an 87-fold molar excess. The final protein concentration was 2 mg/ml. After incubation at room temperature for 60 min, the mixture was treated as above. The resultant protein had spec. act. 59 500 cpm/μg.

2.3. SDS–polyacrylamide gel electrophoresis

Gels (0.5 × 9 cm) were 16% acrylamide, 1% N,N'-diallytartardiamide, 0.2 M glycine (pH 8.6), 0.1% SDS, 0.5 M urea, 0.005 M EDTA, 0.05% TEMED and 0.12% ammonium persulfate. Stacking gels (0.5 cm) consisted of 4% acrylamide, 0.4% DAT, 0.25 M sodium phosphate (pH 6.6), 0.1% SDS, 0.5 M urea, 0.005 M EDTA, 0.1% TEMED and 0.2% ammonium persulfate. Electrophoresis was performed at 2 mA/gel for 2 h followed by 6 mA/gel for 2.5–3 h in a tank buffer of 0.2 M glycine (pH 8.6), 0.1%

SDS, 0.5 M urea and 0.005 M EDTA. The gels were
frozen, sliced into 1.0 mm thick slices and counted
after room temperature incubation in 5 ml 3%
Protosol, 0.4% Omnifluor (NEN) in toluene.

3. Results

3.1. Reductive methylation of IF-3

Our initial attempts at reductive methylation of
IF-3 were carried out according to [1], using [^{14}C]-
formaldehyde and $NaBH_4$. The maximum labeling
achieved was 3300 cpm/μg protein. When $NaBH_3CN$
was substituted as the reducing agent, the resultant
IF-3 had spec. act. >100 000 cpm/μg. IF-3 which is
extensively methylated (>1 methyl group/lysine),
however, was found to be deficient in binding to 30 S
subunits. Only 3–6% of the recovered IF-3 comigrated
with 30 S subunits on sucrose gradients with 25–35%
of the radioactivity present as a more slowly migrating
shoulder (data not shown). This suggested that the
IF-3 has been bound to the ribosomes but that the
interaction was not strong enough to withstand the
pressure of centrifugation.

Therefore we explored various parameters which
would allow us to control the extent of methylation.
The most efficient method proved to be variation of
the concentration of [^{14}C]formaldehyde added as
shown in fig.1. The extent of methylation exhibited
a linear dependence on formaldehyde concentration
up to a ratio of CH_2O/IF-3 = 140. Varying the
CH_2O/$NaBH_3CN$ ratio from 0.28–1.0 resulted in
only a small increase in methylation. Therefore, most
of our subsequent methylations were performed with
equimolar amounts of CH_2O and $NaBH_3CN$, both of
which were present at a 39-fold molar excess over
IF-3. This yielded protein with spec. act. 40 000–
60 000 cpm/μg.

Analysis of the labeled protein by SDS–polyacryl-
amide gel electrophoresis (fig.2) showed that >90%
of the radioactivity migrated as a 22 500 mol. wt
protein, the expected mobility of intact IF-3 [2].
This indicates that the modification procedure did
not result in any appreciable degradation of the
native IF-3.

Such reductively methylated [^{14}C]IF-3 retained
its activity in two functional assays. Binding to 30 S
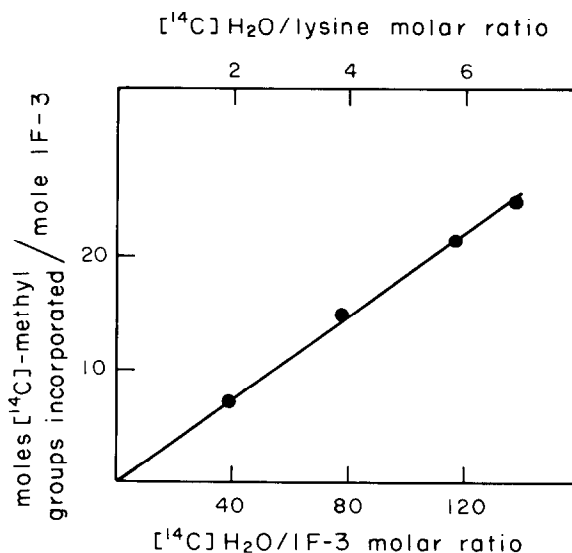subunits, as measured by sucrose density gradient

Fig.1. Effect of formaldehyde concentration on the reductive
methylation of IF-3. Samples contained 9.5 μg IF-3. 0.1%
[^{14}C]formaldehyde (42 mCi/mmol) was added to yield the
indicated molar ratios to IF-3. $NaBH_3CN$ was present in
equimolar amounts with formaldehyde. After 60 min at
room temperature, protein was precipitated with trichloro-
acetic acid, filtered on glass fiber filters and counted. A blank
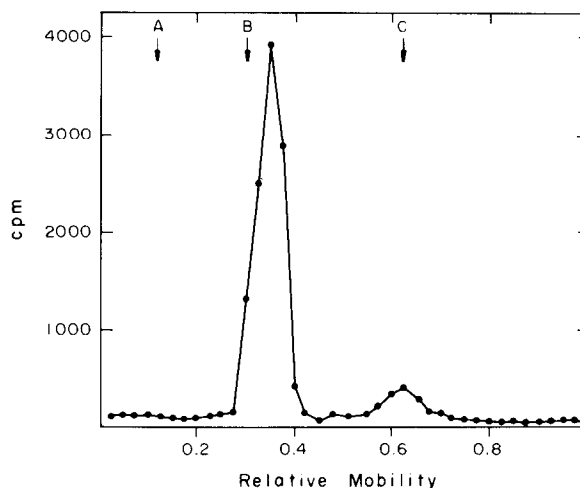lacking IF-3 was subtracted from each determination.

Fig.2. SDS–PAGE analysis of reductively methylated IF-3.
[^{14}C]IF-3 0.32 μg (61 300 cpm/μg) was electrophoresed and
analyzed as in section 2. Arrows indicated the mobility of
marker proteins: (A) ovalbumin (mol. wt 43 000); (B)
α-chymotrypsinogen (mol. wt 25 000); (C) cytochrome c
(mol. wt 12 600).

Table 1
Stimulation of f-met-tRNA binding to 70 S ribosomes by
modified IF-3

Additions	cpm bound	% stimulation
−IF-3	4418	—
+ unmodified IF-3 (2.77 μg)	11 790	2.67
+ [^{14}C]IF-3 (0.83 μg)	7404	1.68
+ [^{14}C]IF-3 (1.65 μg)	9187	2.08
+ [^{14}C]IF-3 (3.3 μg)	11 891	2.69

f-met-tRNA binding to 70 S subunits was performed in a
100 μl mixture containing 50 mM Hepes (pH 7.5), 130 mM
NH_4Cl, 5 mM Mg(OAc)$_2$, 1 mM dithiothreitol, 0.5 mM GTP,
5 μg poly(A,U,G), 0.7 A_{260} units of 70 S ribosomes, 7.5 pmol/
f-[^{3}H]met-tRNA (6600 cpm/pmol), 0.6 μg IF-2 (70% pure)
and varying amounts of IF-3 (0.8–4.0 μg). The mixture,
lacking tRNA and the initiation factors, was preincubated at
37°C for 15 min to activate the ribosomes [4] and, after
addition of the other components, incubation was continued
for 15 min at 25°C and the binding measured by adsorption
to nitrocellulose filters

centrifugation, was normal. Of the input IF-3
30–40% was bound to 30 S subunits when both
were present at equimolar concentrations and this
binding was saturable, leveling off at 1.07 mol IF-3
bound/mol 30 S subunits at an IF-3/30 S input ratio
of 2.5 (data not shown). This modified IF-3 was
nearly as active as control, unmethylated IF-3 in
stimulating the binding of f-met-tRNA to 70 S ribo-
somes in the presence of IF-2 (table 1).

3.2. Reductive methylation of EFTu · GDP

A similar methylation procedure as described for
IF-3 was applied to EFTu · GDP except that in this
case the pH was 9.0 (the EFTu was to be used in sub-
sequent assays which required pH 9). Once again,
much higher labeling was achieved in the presence
of NaBH$_3$CN as the reducing agent, 60 000 cpm/μg
as compared to 6000 cpm/μg with NaBH$_4$. The
methylated EFTu is highly active in stimulating
enzymatic binding of the phe-tRNA to 70 S ribo-
somes (fig.3).

4. Discussion

Both IF-3 and EFTu have been labeled before
with [^{14}C]formaldehyde and NaBH$_4$ [5–9]. In [7]
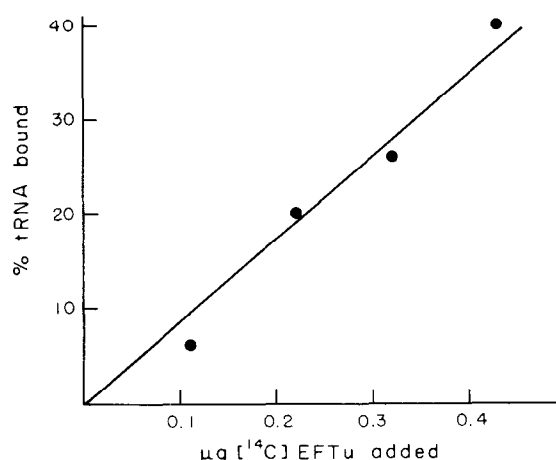the maximum labeling of IF-3 was 21 150 cpm/μg.

Fig.3. Stimulation of phe-tRNA binding to the ribosomal A
site by [^{14}C]EFTu. EFTu-dependent binding of phe-tRNA
to ribosomes was measured in a 100 μl mixture containing
2.5 pmol [^{3}H]phe-tRNA, 0.9 A_{260} 70 S ribosomes, 5 μg
poly(U) and EFTu · GDP as indicated in 50 mM Hepes
(pH 7.4), 75 mM NH$_4$Cl, 75 mM KCl, 8 mM MgCl$_2$, 1 mM
dithiothreitol, and 0.5 mM GTP. Ribosomes were activated
by preincubation at 37°C for 15 min before the addition of
EFTu to tRNA. 0.56 A_{260} units of crude tRNA were added
prior to the phe-tRNA in order to block EFTu-independent
binding. Mixtures were then incubated at 30°C for 25 min
and the binding measured by adsorption to nitrocellulose
filters.

This can be increased at least 3-fold by the procedure
described here without affecting the function of
IF-3. Such IF-3 contains 16 mol methyl groups/mol
protein. Reductive alkylation with formaldehyde and
NaBH$_4$ favors dimethyllysine formation [10] but at
low excess of NaBH$_3$CN to amine the subsequent
reaction with a second carbonyl group is not favored
[11]. Therefore, it is not clear whether the reaction
described represents the modification of 8 lysine
residues 16 lysine residues or some intermediate
number.

It is interesting to note that IF-3 labeled above
100 000 cpm/μg had altered affinity for the 30 S
subunit. Since the pK of mono- and di-methyllysine
is only slightly altered from that of lysine [10] the
modification should not affect any electrostatic
interaction between IF-3 and the ribosome. Similarly,
because of the small size of the methyl group it does
not seem likely that steric hindrance of the interac-
tion occurred. It is thus tempting to speculate that
extensive modification interferes with a hydrogen

bonding interaction between some lysines in IF-3 and appropriate acceptors on the ribosome.

EFTu also retained its functional activity after extensive modification. The extent of ^{14}C-labeling described here represents a 4-fold increase over that obtained in [9]. EFTu at 59 500 cpm/μg has 29.5 mol methyl group/mol protein and this represents 74% of the maximum possible labeling (assuming complete dimethylation of all lysines; EFTu contains 20 Lys/ molecule [12]). It was shown [13] that reductively methylated EFTu (using formaldehyde and NaB[^{3}H]$_4$) was active in stimulating polyphenylalanine synthesis and we confirm that its ability to stimulate binding of aa-tRNA to the A site is unaltered.

NaBH$_3$CN has been used in the reductive methylation of proteins. It was found [14] that the reductive alkylation of BSA at pH 7.2 showed a linear dependence on the aldehyde concentration (in this case pyridoxal) but that a similar variation in the NaBH$_3$CN concentration had little effect. We have confirmed this behavior in the case of IF-3 alkylation (fig.1). This is to be expected based on the proposed mechanism of the reaction [10]. Since formation of the Schiff's base is the slow step, an increase in the aldehyde concentration would be expected to result in greater modification. Reduction, on the other hand, occurs very rapidly and should only require stoichiometric amounts of NaBH$_3$CN. The reductive methylation of a series of proteins with [^{14}C]CH$_2$O and NaBH$_3$CN was reported [15] but no data on the subsequent functional activity of such extensively methylated proteins was presented. The experiments presented here not only confirm the labeling technique but demonstrate that the activity of the modified proteins can be maintained.

The substitution of NaBH$_3$CN for NaBH$_4$ as the reducing agent has a number of advantages.

(1) The reaction can be performed at neutral pH, rather than pH 9 or above. This is so because NaBH$_3$CN is stable down to pH 3–4 [11] unlike NaBH$_4$ which is unstable at neutral pH. Since formation of Schiff's base is maximal at pH 6 (albeit unfavored) [11] a lower pH enhances the extent of methylation.

(2) Smaller amounts of CH$_2$O are required for the methylation. This is again a reflection of the greater stability of formaldehyde at neutral pH. Our conditions for the methylation of IF-3 (to

60 000 cpm/μg) required only a 2-fold molar excess of CH$_2$O over lysine as opposed to that in [1] which uses a 5-fold excess and only results in protein with, at best, 33% of the specific radioactivity.

(3) Much higher levels of labeling can be achieved with NaBH$_3$CN and this procedure can be applied to a wide variety of proteins without any major effect on function.

Acknowledgements

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