再構成型無細胞タンパク質合成系(PURE*frex*®)の酸化還元状態の解析

Analysis of the redox state in a reconstituted cell-free protein synthesis system (PURE*frex*®)

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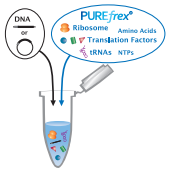
Abstract

PURE*frex*は、タンパク質合成に関与する因子のみから再構成した無細胞タンパク質合成系であり、現行のPURE*frex*には、サイトゾル環境を模して還元状態で反応させるため、還元剤としてジチオスレイトール(DTT)を添加している。ところが、長時間インキュベートした場合に、反応液が酸化状態になっていることが確認された。具体的には、DNAを含まないPURE*frex*反応液を37℃で24時間インキュベーション後、還元剤を添加しないSDS-PAGEで解析すると、EF-Tuなど一部の翻訳因子で移動度が変化したバンドが出現した。そこで、インキュベーション後の反応液を質量分析で解析したところ、翻訳因子のシステイン残基に遊離のシステインが付加したと推定されるペプチドが検出された。また、エルマン試薬を用いたSH基量の定量により、24時間インキュベートした後の反応液ではSH基が検出限界程度まで減少していることが分かった。これらの現象は、還元剤として還元型グルタチオン(GSH)を使用した場合には観察されなかった。添加する還元剤を変えてタンパク質合成を行ったところ、合成量はほとんど変化しなかったが、合成タンパク質のジスルフィド結合形成に影響を与えていた。以上の結果より、PURE*frex*でタンパク質を合成する際には、合成するタンパク質に適切な還元剤を選択する必要があることが明らかになった。

1. PURE*frex*®

PURE*frex* is based on the PURE system. The PURE system is a reconstituted cell-free protein synthesis system, which consists of only purified factors necessary for transcription, translation and energy regeneration.



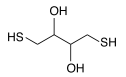
Advantage

- Low level of contamination
- Easy adjustment of the reagent composition
- PCR product usable as a template DNA

(Ref; Shimizu Y. *et al.* (2001) *Nat. Biotechnol.*, vol. 19, p. 751)

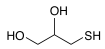
2. Reducing reagents used in this research

Dithiothreitol (DTT)



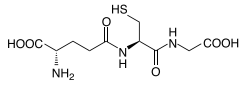
2-Mercaptoethanol (2ME)

3-Mercapto-1,2-propanediol (MPD)

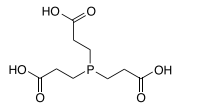


Cysteine (Cys)

Reduced Glutathione (GSH)

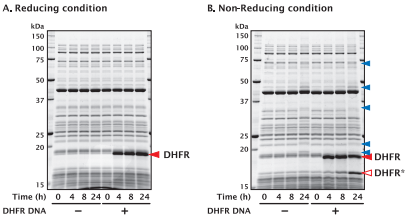


Tris(2-carboxyethyl)phosphine (TCEP)



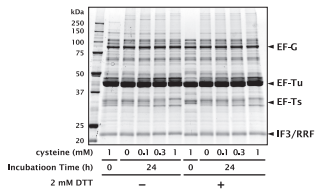
3. Oxidation and cysteinylation of proteins in the current PURE*frex* during long incubation

PURE*frex*®2.0
↓ +/- DHFR DNA
↓ incubation at 37ºC for 0, 4, 8 and 24 h
↓ SDS-PAGE under reducing and non-reducing condition



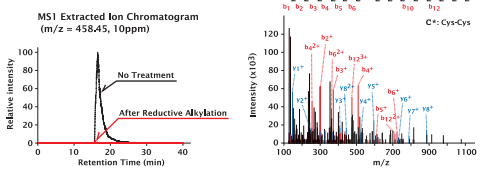
Several proteins in the current PURE*frex* were oxidized after long incubation.

PURE*frex*®2.0 Solution II (Protein Mix)
↓ + Solution I (Buffer Mix) containing 0, 0.1, 0.3 or 1 mM Cys and 0 or 2 mM DTT
↓ incubation at 37ºC for 0 and 24 h
↓ SDS-PAGE under non-reducing condition

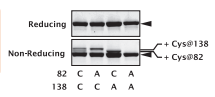


The amount of oxidized proteins was dependent on the concentration of cysteine.

PURE*frex*®2.0 Solution II (Protein Mix)
↓ + Solution I containing 1 mM Cys and 2 mM DTT
↓ incubation at 37ºC for 24 h
↓ +/- reductive alkylation
↓ MS/MS analysis

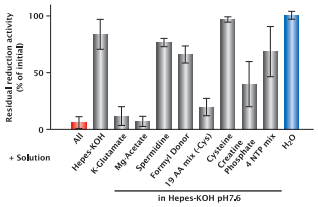


EF-Tu (WT, C82A, C138A, C82A/C138A)
↓ + Solution I containing 1 mM Cys and 2 mM DTT
↓ incubation at 37ºC for 24 h
↓ SDS-PAGE under reducing and non-reducing condition



Two cysteines of EF-Tu were cysteinylated after incubation with Solution I.

2 mM DTT
↓ + each component in Solution I
↓ incubation at 37ºC for 24 h
↓ Ellman' s assay

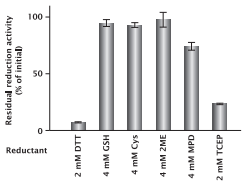


Reduction activity of DTT was almost depleted after incubation with K-Glutamate and Mg-Acetate.

4. Comparison of reducing reagents

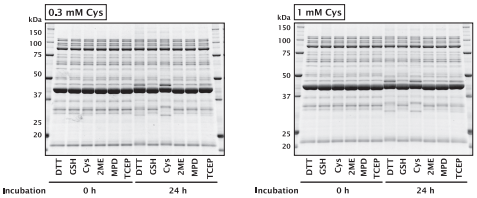
4-1. Influence on the components of PURE*frex*

Indicated reducing reagent
↓ + Solution I (-DTT)
↓ incubation at 37ºC for 24 h
↓ Ellman' s assay



Reduction activity of GSH, Cys or 2ME did not change after incubation with Solution I.

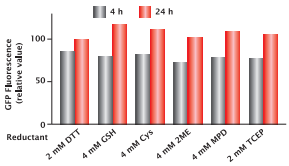
PURE*frex*®2.0 Solution II (Protein Mix)
↓ + Solution I (Buffer Mix) containing indicated reducing reagent and 0.3 or 1 mM Cys
↓ incubation at 37ºC for 0 and 24 h
↓ SDS-PAGE under non-reducing condition



Most of protein components were not oxidized after incubation with Solution I containing GSH, 2ME, or MPD.

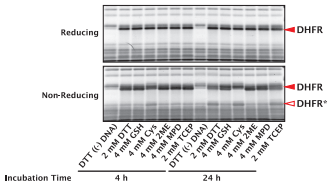
4-2. Synthesis of proteins without a disulfide bond

PURE*frex*®2.0 containing indicated reducing reagent
↓ + GFP DNA
↓ incubation at 37ºC for 4 and 24 h
↓ measurement of fluorescence of syntheiszed GFP



Fluorescence of synthesized GFP was same regardless of reducing reagents.

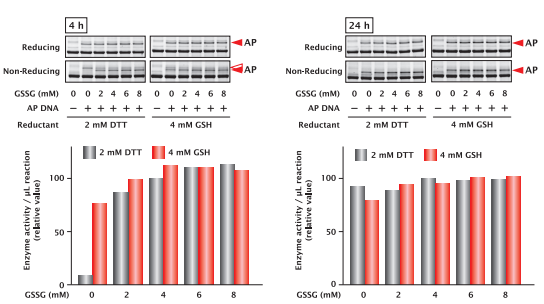
PURE*frex*®2.0 containing indicated reducing reagent
↓ +/- DHFR DNA
↓ incubation at 37ºC for 4 and 24 h
↓ SDS-PAGE under reducing and non-reducing condition



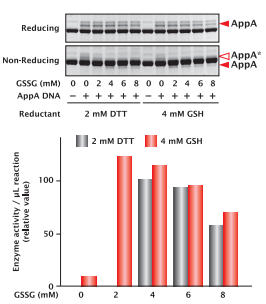
Synthesized DHFR was less oxidized in case of using GSH, 2ME and MPD.

4-3. Synthesis of proteins containing disulfide bonds

PURE*frex*®2.0 containing DTT or GSH
↓ + oxidized glutathione (GSSG) with indicated concentration
↓ +/- Alkaline phosphatase (AP) DNA
↓ incubation at 37ºC for 4 and 24 h
↓ SDS-PAGE under reducing and non-reducing condition
AP enzymatic activity assay

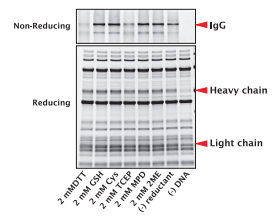


PURE*frex*®2.0 containing DTT or GSH
↓ + GSSG and 4 µM DsbC
↓ +/- Acid phosphatase (AppA) DNA
↓ incubation at 37ºC for 4 h
↓ SDS-PAGE under reducing and non-reducing condition
AppA enzymatic activity assay



Reducing reagents influenced on the formation of correct disulfide bonds within and between synthesized proteins

PURE*frex*®2.0 containing indicated reducing reagent
↓ +/- Trastuzumab HC and LC DNA
↓ incubation at 37ºC for 16 h
↓ SDS-PAGE under reducing and non-reducing condition



Summary

- Several proteins in the current PURE*frex* containing DTT were oxidized and cysteinylated after long incubation.
- Influence on the components of PURE*frex* was dependent on reducing reagent.
- Efficiency of protein synthesis was almost same regardless of reducing reagents.
- Reducing reagents influenced on the formation of correct disulfide bonds within and between synthesized proteins.

Suitable reducing reagent is to be selected according to a target protein.