Efficient introduction of aryl bromide functionality into proteins in vivo

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Abstract Artificial proteins can be engineered to exhibit interesting solid state, liquid crystal or interfacial properties and may ultimately serve as important alternatives to conventional polymeric materials. The utility of protein-based materials is limited, however, by the availability of just the 20 amino acids that are normally recognized and utilized by biological systems; many desirable functional groups cannot be incorporated directly into proteins by biosynthetic means. In this study, we incorporate *para*-bromophenylalanine (*p*-Br-phe) into a model target protein, mouse dihydrofolate reductase (DHFR), by using a bacterial phenylalanyl-tRNA synthetase (PheRS) variant with relaxed substrate specificity. Coexpression of the mutant PheRS and DHFR in a phenylalanine auxotrophic *Escherichia coli* host strain grown in *p*-Br-phe-supplemented minimal medium resulted in 88% replacement of phenylalanine residues by *p*-Br-phe; variation in the relative amounts of phe and *p*-Br-phe in the medium allows control of the degree of substitution by the analog. Protein expression yields of 20–25 mg/l were obtained from cultures supplemented with *p*-Br-phe; this corresponds to about two-thirds of the expression levels characteristic of cultures supplemented with phe. The aryl bromide function is stable under the conditions used to purify DHFR and creates new opportunities for post-translational derivatization of brominated proteins via metal-catalyzed coupling reactions. In addition, bromination may be useful in X-ray studies of proteins via the multiwavelength anomalous diffraction (MAD) technique.

Key words: Amino acid analog; *Para*-bromophenylalanine; Multiwavelength anomalous diffraction; Phenylalanyl-tRNA synthetase; Phenylalanine replacement; Protein engineering

1. Introduction

Protein engineering provides a powerful tool for modification of the structural and functional properties of proteins as well as for designing new macromolecular substances with precise control of composition and architecture. Several polymeric materials with interesting properties have been synthesized by protein engineering methods [1–4]. For many applications of these macromolecules, it will be desirable to develop methods for incorporation of amino acids containing novel chemical functionality not possessed by the 20 proteinogenic amino acids. New functionality can be exploited to probe protein structure and function, to alter stability and folding behavior, and to open new avenues for post-translational modification.

Biosynthetic incorporation of non-canonical amino acids into proteins has been achieved in *Escherichia coli* largely by exploiting the capacity of the wild-type protein synthesis apparatus to utilize analogs of the natural amino acids [5–11]. However, use of a wider range of analogs will require that the substrate specificity of the aminoacyl-tRNA synthetases (aaRS) is relaxed or changed altogether. The in vitro method of unnatural amino acid incorporation, introduced by Hecht and coworkers [12], uses chemically acylated tRNAs and thereby circumvents the checkpoint of the aaRS; this method has found extensive application in amino acid replacement in a site-specific manner [13–15]. A further advance in site-specific replacement was the demonstration of in vivo incorporation of an amino acid analog by using a yeast suppressor $tRNA_{amber}$/aaRS pair in an analog-resistant *E. coli* strain [16].

We have previously exploited the ability of *E. coli* to incorporate *para*-fluorophenylalanine (*p*-F-phe) into repetitive polypeptides with high efficiency [10]. While fluorinated polypeptide materials are likely to exhibit novel surface properties, they are inert to chemical modification under mild conditions. We therefore turned our attention to *para*-bromophenylalanine (*p*-Br-phe), which we anticipate will be readily modified owing to the higher reactivity of the aryl bromide functional group [17–22]. Moreover, bromination may be useful in X-ray structure determination of proteins by multiwavelength anomalous diffraction (MAD). Brominated nucleic acids have been used successfully for determination of the structures of DNA–drug complexes, DNA–protein complexes, DNAs and RNAs [23–27]. We suggest that *p*-Br-phe may eventually complement selenomethionine [28,29] in structure determination of proteins by the MAD technique.

While the wild-type *E. coli* protein synthesis machinery can utilize phenylalanine (phe) and *p*-F-phe, the analog *p*-Br-phe is not recognized by the wild-type phenylalanyl-tRNA synthetase (PheRS). In 1991, however, Kast and Hennecke reported an engineered mutant form of this enzyme (Gly294PheRS) which charges $tRNA^{Phe}$ with *p*-Cl-Phe in vivo and appears to enable incorporation of even *p*-Br-phe into cellular protein [30–32]. In the present work, we employed this mutant to effect efficient in vivo incorporation of *p*-Br-phe into mouse dihydrofolate reductase (DHFR) in bacterial cells.

2. Materials and methods

2.1. Plasmids and E. coli strains

The plasmid pKSS bears the gene for the Gly-294 variant α-subunit of the PheRS (termed *pheS**) [33]. *pheS** was subcloned from pKSS

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by PCR mutagenesis such that two *Sph*I restriction sites, one at either end outside of the *pheS** gene, were generated by single-base insertions (*Sph*I sites were designed for another purpose). The 1722 bp PCR-amplified fragment was cloned into a pUC19 cloning vector at the *Sma*I site. The *pheS** gene was cut out as an *Sph*I fragment, blunt-ended, and inserted into the *Pvu*II site of the expression plasmid pQE15 (Qiagen). The resulting plasmid, designated pQE-FS, carries the gene for the target protein DHFR under bacteriophage T5 promoter control and that for *pheS** under *lac* promoter control. The repressor plasmid pLysS (Novagen) was modified so that it contained the *lacI*^{q} gene for *lac* repressor as a *Sal*I fragment from the plasmid pREP4 (Qiagen); the resulting plasmid was designated pLysS-IQ.

A phenylalanine auxotrophic derivative of *E. coli* strain BL21(DE3), called AF, previously constructed in our laboratory, was used as host [10]. The AF strain with the repressor plasmid pLysS-IQ was designated AF-IQ. AF-IQ cells were transformed with pQE-FS or with control plasmid pQE15.

2.2. Gene expression and incorporation of p-Br-phe into DHFR

The in vivo translational activity of *p*-Br-phe was first investigated on a small scale. Bacterial cultures were grown in M9 minimal medium [34] supplemented with glucose, thiamine, $MgSO_4$, $CaCl_2$, 19 amino acids (at 20 mg/l), antibiotics (ampicillin and chloramphenicol) and phenylalanine (at 20 mg/l). AF-IQ[pQE15] and AF-IQ[pQE-FS] cultures were grown in supplemented M9 medium containing phe. At an optical density at 600 nm (OD_{600}) of 0.8–1.0, cells were sedimented by centrifugation, washed twice with 0.9% NaCl and resuspended in supplemented M9 medium containing either: (i) 500 mg/l *p*-Br-phe, (ii) 20 mg/l phe (positive control) or (iii) no phe or analog (negative control). Gene expression was induced 10 min after the medium shift by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were harvested 4 h post-induction and protein expression was monitored by analysis of whole cell lysates on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE).

Larger scale production of DHFR was carried out in 1 l cultures of AF-IQ[pQE-FS] grown in the medium described above. Cells were sedimented and the cell pellet was resuspended in supplemented M9 medium containing *p*-Br-phe at 500 mg/l; expression of DHFR was induced 10 min after the medium shift with 1 mM IPTG. In experiments to control the degree of bromination of DHFR, the induction medium was supplemented with varying amounts of phe and *p*-Br-phe (see Table 1).

2.3. Isolation and analysis of brominated target protein

DHFR as expressed from pQE15-derived plasmids contains an N-terminal hexahistidine sequence, which was utilized for protein purification by nickel-affinity chromatography with step-wise pH gradient elution under denaturing conditions (Qiagen). Eluted protein was dialyzed against distilled water and lyophilized, and the purified protein was subjected to amino acid compositional analysis.

2.4. Extraction of cellular free amino acids

In order to determine the cellular concentrations of free phe and *p*-Br-phe, 250 ml cell cultures were grown in M9 medium supplemented with all 20 amino acids at 20 mg/l. The cells were harvested at $OD_{600} = 1$, washed twice with 0.9% NaCl and treated as follows: (i) subjected to analysis as such, or (ii) incubated for 2 h in M9 medium lacking phe and containing IPTG, *p*-Br-phe, both or neither as described in Fig. 2. Free amino acids were extracted by a modified version of the protocol of Raunio and Rosenqvist [35]; harvested cells were resuspended in 5 ml distilled water, and the suspension adjusted to pH 10 with a few drops of 5 N NH_4OH. The suspension was incubated without shaking for 10 min at 100°C in a water bath. After cooling, the pH was adjusted to about 2 by addition of HCl. The precipitate thus obtained was removed by centrifugation at $4000 \times g$ for 10 min at 4°C. The supernatant was frozen and a 100 μl aliquot was lyophilized and subjected to amino acid compositional analysis. Similar experiments were conducted using M9 medium supplemented (at 20 mg/l) with just the three amino acids that are essential for growth of the AF strain viz. proline, arginine and phenylalanine.

3. Results

3.1. Gly294PheRS allows p-Br-phe incorporation into proteins

An AF-IQ[pQE-FS] culture was grown in supplemented M9 minimal medium to an OD_{600} of 0.8–1.0, and then shifted to a medium containing either (i) *p*-Br-phe, (ii) phe (positive control) or (iii) no phe or *p*-Br-phe (negative control). Expression of DHFR was induced for 4 h with 1 mM IPTG. A control expression experiment was done with AF-IQ[pQE15], which contains only the wild-type PheRS. No expression of DHFR was observed in the negative control cultures or in the AF-IQ[pQE15] culture containing *p*-Br-phe. On the other hand, AF-IQ[pQE-FS] cultures supplemented with *p*-Br-phe expressed DHFR (albeit at lower levels than the positive controls), as noted from SDS–PAGE analysis of the respective whole cell lysates (Fig. 1). The target proteins were purified by nickel-affinity chromatography. The yield of purified target protein isolated from AF-IQ[pQE-FS] cells grown in *p*-Br-phe-supplemented medium (DHFR-Br) was 20–25 mg/l, which is approximately 70% of the yield of DHFR obtained from the same cells grown in medium supplemented with phe. Amino acid analysis of purified DHFR-Br indicated up to 88% substitution of phe residues (there are nine phe residues in DHFR) by *p*-Br-phe. The decrease in phe content was compensated by an increase in *p*-Br-phe content, indicating that *p*-Br-phe is stable under the conditions of protein purification and amino acid analysis.

3.2. Control of degree of substitution

For many purposes of post-translational chemical modification of proteins, complete substitution by the analog may not be necessary (or may even be detrimental). We explored the possibility of gaining control over *p*-Br-phe incorporation

Table 1
Control of the level of *p*-Br-phe incorporation

Experiment	*p*-Br-phe (mg/l)	Phe (mg/l)	Yield of DHFR (mg/l culture)	*p*-Br-phe (%)
1	0	20	36	0
2	250	20, no media shift	55	10
3	500	20, no media shift	30	10
4	500	8, no media shift	28	60
5	250	0	20	46
6	500	0	24	88
7	750	0	24	87
8	1000	0	22	78
9	500	1	28	70
10	500	2	30	65
11	250	2	30	20

Cells were grown to an OD_{600} of 1, harvested, washed twice and then shifted to medium containing the indicated amounts of phe and *p*-Br-phe. No media shift indicates that the cells were not washed and in that case, phe indicates the initial concentration supplied at the start of the culture.

by supplementing the medium with varying amounts of phe and *p*-Br-phe during target protein expression. AF-IQ[pQE-FS] cells were grown in M9 medium as before and shifted to media containing the amounts of phe and *p*-Br-phe indicated in Table 1. The results shown in Table 1 compare the levels of *p*-Br-phe incorporation and the yields of protein obtained for different concentrations of phe and *p*-Br-phe. The bromination level increases with increasing concentrations of *p*-Br-phe up to 500 mg/l; higher concentrations of *p*-Br-phe do not change the degree of incorporation further under our conditions (experiments 5–8). Addition of phe along with *p*-Br-phe improves the yield of protein modestly while reducing the degree of bromination as expected (experiments 9–11). Addition of *p*-Br-phe to growing cultures at the time of induction without the removal of phe resulted in very low levels of incorporation of the analog when the initial phe concentration was high (i.e. 20 mg/l). When the culture medium initially contained only 8 mg/l of phe, the level of substitution increased to about 60% (experiments 2–4).

3.3. Determination of free amino acids in the cell

In an attempt to understand why we did not observe complete replacement of phe by *p*-Br-phe, we determined the intracellular concentrations of free phe and *p*-Br-phe in AF-IQ[pQE-FS] cells under different growth conditions. We were particularly interested in the possibility that starvation for phe, induction of the T5 promoter and subsequent overexpression of DHFR, addition of *p*-Br-phe or a combination of these events might lead to a stress response and enhance cellular protein turnover, thereby increasing the cellular pool of phe. Cells were grown in the presence of phe, washed twice with saline and then grown in the presence of IPTG, *p*-Br-phe, or both, as described in Section 2. After 2 h of incubation, the amounts of free cellular phe and *p*-Br-phe were determined. The results in Fig. 2 indicate that the phe concentration does not vary significantly under the conditions examined and remains at 0.65 ± 0.15 μM, significantly lower than that supplied to the medium (120 μM). The concentration of cellular *p*-Br-phe was found to be 14–17 times higher than that of phe, but remained in the micromolar range even when the amount supplied to the culture medium was in the millimolar range. Similar results were obtained when cells were grown in the presence of just the three amino acids essential for growth of the AF strain. Incomplete replacement of phe thus appears to be a simple consequence of the fact that the medium shift does not remove the last traces of the natural amino acid from the culture.

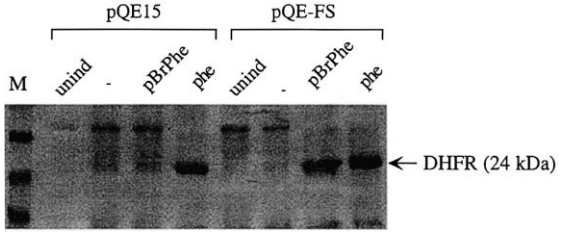

Fig. 1. *pheS** allows utilization of *p*-Br-phe as a substrate for PheRS. Production of DHFR in the *p*-Br-phe-supplemented medium is observed only from plasmid pQE-FS wherein the gene for the mutant enzyme with relaxed substrate specificity, *pheS**, is present. Cells transformed with pQE15 lack the mutant enzyme and cannot utilize *p*-Br-phe to make DHFR. In the negative control, which contains neither phe nor *p*-Br-phe (lanes marked −), there is no DHFR, confirming auxotrophy for phenylalanine. Concentration of phe = 20 mg/l; *p*-Br-phe = 500 mg/l.

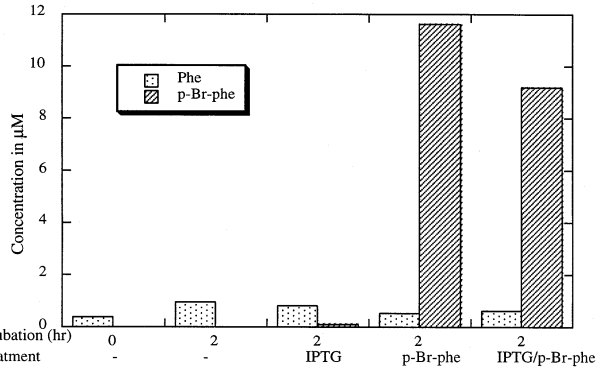

Fig. 2. Cellular concentrations of phenylalanine and *p*-Br-phe in *E. coli* strain AF-IQ[pQE-FS]. Cells were grown to an $OD_{600} = 1$, centrifuged, washed twice with NaCl and subjected to treatment as indicated above. Concentration of IPTG = 1 mM; *p*-Br-phe = 2 mM.

4. Discussion

Incorporation of *p*-Br-phe into short peptides by solid state peptide synthesis has been reported previously [36–38]. The results reported here constitute the first demonstration of an efficient strategy for incorporation of *p*-Br-phe into protein in vivo. This was achieved by coexpression of the gene for a PheRS variant with relaxed substrate specificity, along with the gene encoding the protein of interest, in an *E. coli* host strain auxotrophic for phenylalanine. The *E. coli* strain we use still expresses its wild-type PheRS α-subunit; however, since the mutant PheRS α-subunit is produced from several plasmid-borne gene copies, most functional PheRS molecules (which have the oligomeric structure $[\alpha\beta]_2$ [39,40]) are likely to contain the mutant subunit and thus possess the ability to utilize *p*-Br-phe. High levels of replacement (88%) of phenylalanine by its brominated analog have been achieved. The brominated analog is stable once incorporated into protein, and the yield of brominated protein is high enough for sample-intensive methods such as post-translational modification, nuclear magnetic resonance or crystallographic structure determination.

Controlling the amount of *p*-Br-phe substitution may be advantageous for many applications, e.g. post-translational modification, as it may not be necessary to replace all of the phenylalanine residues by the brominated analog. The level of analog incorporation can be controlled by varying the relative amounts of phe and *p*-Br-phe in the culture medium at the time of induction of target gene expression. The success shown here with the model protein (DHFR) is likely to be applicable to other proteins as well.

The in vivo method for unnatural amino acid incorporation described here is complementary to the in vitro approach, which can accommodate a wider range of analogs at a single programmed site using chemically misacylated tRNAs [12–14]. The in vivo approach, on the other hand, is best suited for substitution by an analog at multiple sites and for obtaining higher protein yields. The method described here allows routine isolation of tens to hundreds of mg of labeled protein within days at reasonable cost.

Introduction of aryl bromide functionality opens possibilities for a variety of controlled chemical modifications of engineered proteins, many of which are not possible with the 20 natural amino acids. Aromatic halides are known to undergo metal-catalyzed coupling, amidation, cyanation and related reactions, under mild conditions [17–22]. Application of these chemistries to genetically engineered proteins would allow for new approaches to side-chain modification, coupling with various ligands, immobilization on surfaces, and synthesis of graft copolymers and cross-linked gels. Bromination may also provide a new tool for use in X-ray diffraction studies of protein structure, though we do not yet know the extent to which *p*-Br-phe residues may perturb protein folding. Brominated pyrimidine bases have been successfully used as anomalous scatterers for structure determination of DNA–drug complexes, DNA–protein complexes and other nucleic acids [23–27]. We suggest that *p*-Br-phe is likely to find similar uses in crystallographic studies of protein structure.

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References

[1] Krejchi, M.T., Atkins, E.D.T., Waddon, A.J., Fournier, M.J., Mason, T.L. and Tirrell, D.A. (1994) Science 265, 1427–1432.
[2] Yu, S.J.M., Conticello, V.P., Zhang, G.H., Kayser, C., Fournier, M.J., Mason, T.L. and Tirrell, D.A. (1997) Nature 389, 167–170.
[3] Petka, W.A., Harden, J.L., McGrath, K.P., Wirtz, D. and Tirrell, D.A. (1998) Science 281, 389–392.
[4] Panitch, A., Yamaoka, T., Fournier, M.J., Mason, T.L. and Tirrell, D.A. (1999) Macromolecules 32, 1701–1703.
[5] Richmond, M.H. (1962) Bacteriol. Rev. 26, 398–420.
[6] Ibba, M. (1996) Biotechnol. Genet. Eng. 13, 197–216.
[7] Deming, T.J., Fournier, M.J., Mason, T.L. and Tirrell, D.A. (1997) J. Macromol. Sci. Pure Appl. Chem. A34, 2143–2150.
[8] Kothakota, S., Mason, T.L., Tirrell, D.A. and Fournier, M.J. (1995) J. Am. Chem. Soc. 117, 536–537.
[9] van Hest, J.C.M. and Tirrell, D.A. (1998) FEBS Lett. 428, 68–70.
[10] Yoshikawa, E., Fournier, M.J., Mason, T.L. and Tirrell, D.A. (1994) Macromolecules 27, 5471–5475.
[11] Dougherty, M.J., Kothakota, S., Mason, T.L., Tirrell, D.A. and Fournier, M.J. (1993) Macromolecules 26, 1779–1781.
[12] Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. and Schultz, P.G. (1989) Science 244, 182–188.
[13] Heckler, T.G., Roesser, J.R., Xu, C., Chang, P. and Hecht, S.M. (1988) Biochemistry 27, 7254–7262.
[14] Hecht, S.M. (1992) Acc. Chem. Res. 25, 545–552.
[15] Cornish, V.W., Mendel, D. and Schultz, P.G. (1995) Angew. Chem. Int. Ed. Engl. 34, 621–633.
[16] Furter, R. (1998) Prot. Sci. 7, 419–426.
[17] Schoenberg, A. and Heck, R.F. (1974) J. Org. Chem. 39, 3327–3331.
[18] Sakakibara, Y., Ido, Y., Sasaki, K., Sakai, M. and Uchino, N. (1993) Bull. Chem. Soc. Jpn. 66, 2776–2778.
[19] Nguefack, J., Bolitt, V. and Sinuo, D. (1996) Tetrahedron Lett. 37, 5527–5530.
[20] Riguet, E., Alami, M. and Caheiz, G. (1997) Tetrahedron Lett. 38, 4397–4400.
[21] Yoneyama, M., Kakimoto, M. and Imai, Y. (1988) Macromolecules 21, 1908–1911.
[22] Yoneyama, M., Konishi, T., Kakimoto, M. and Imai, Y. (1990) Macromol. Chem. Rapid Commun. 11, 381–386.
[23] Correll, C.C., Freeborn, B., Moore, P.B. and Steitz, T.A. (1997) Cell 91, 705–712.
[24] Correll, C.C., Freeborn, B., Moore, P.B. and Steitz, T.A. (1997) J. Biomol. Struct. Dyn. 15, 165–172.
[25] Shah, S.A. and Brunger, A.T. (1999) J. Mol. Biol. 285, 1577–1588.
[26] Shepard, W., Cruse, W.B.T., Forume, R., Fortelle, E. and Prange, T. (1998) Structure 6, 849–861.
[27] Hendrickson, W.A. (1999) J. Synchrotron Rad. 6, 845–851.
[28] Hendrickson, W.A., Horton, J.R. and LeMaster, D.M. (1990) EMBO J. 9, 1665–1672.
[29] Wei, Y., Hendrickson, W.A., Crouch, R.J. and Satow, Y. (1990) Science 249, 1398–1405.
[30] Kast, P. and Hennecke, H. (1991) J. Mol. Biol. 222, 99–124.
[31] Ibba, M., Kast, P. and Hennecke, H. (1994) Biochemistry 33, 7107–7112.
[32] Ibba, M. and Hennecke, H. (1995) FEBS Lett. 364, 272–275.
[33] Kast, P. (1994) Gene 138, 109–114.
[34] Miller, J.H. (1992) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[35] Raunio, R. and Rosenqvist, H. (1970) Acta Chem. Scand. 24, 2737–2744.
[36] Hruby, V.J., Bartosz-Bechowski, H., Davis, P., Slaininova, J., Zalewska, T., Stropova, D., Porreca, F. and Yamamura, H.I. (1997) J. Med. Chem. 40, 3957–3962.
[37] Nose, T., Fujita, T., Nakajima, M., Inoue, Y., Costa, T. and Shimogihashi, Y. (1998) J. Biochem. 124, 354–358.
[38] Saito, N.G., Chang, H.-C. and Paterson, Y. (1999) J. Immunol. 162, 5998–6008.
[39] Mosyak, L. and Safro, M. (1993) Biochimie 75, 1091–1098.
[40] Mosyak, L., Reshetnikova, L., Goldgur, Y., Delarue, M. and Safro, M.G. (1995) Nat. Struct. Biol. 2, 537–547.