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Abstract

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Incorporation of chlorinated analogues of aliphatic amino acids during cell-free protein synthesis†‡

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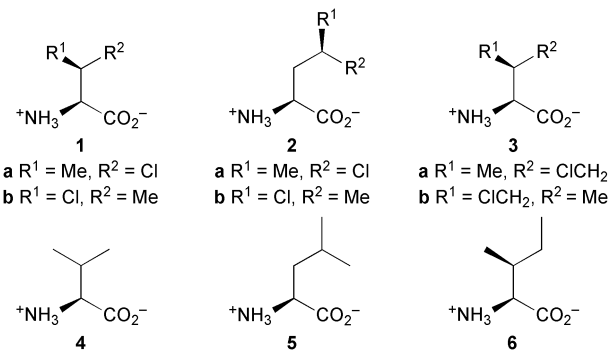
3-Chloro-Abu and 4-chloro-Nva are biosynthetically incorporated into *E. coli* peptidyl-Pro *cis-trans* isomerase B, as substitutes for Val and Leu, respectively. The extent of incorporation is up to ~90%, and substituted protein is catalytically active. By contrast, 4-chloro-Val is not an effective replacement for Ile.

While protein biosynthesis typically employs only 20 amino acids, many others are also incorporated by native biosynthetic machinery.[1–3] Generally, each of the others has a structure closely related to one of the 20 and therefore it competes with the normal substrate to be loaded onto the corresponding tRNA by the cognate aminoacyl-tRNA synthetase. Cell-free protein production systems provide the opportunity to bias this competition.[1,4] These systems employ cell extracts as the source of the ribosomes, aminoacyl-tRNA synthetases and translation factors, combined with a medium containing the other ingredients required for protein synthesis. Excluding one of the normal amino acid substrates from the medium, and adding a structurally analogous species instead, increases the likelihood of incorporation of the analogue. Auxotrophic cell strains which require exogenous supply of one or more of the normal amino acids can also enable incorporation of analogues.[2] These approaches have been used for the synthesis of proteins containing high levels of fluorinated,[5] dehydro[6] and isotopically-labelled[7,8] amino acids, as well as 3,4-dihydroxy-Phe[9] and seleno-Met[10] in place of Tyr and Met, respectively.

Our interest in chlorinated aliphatic amino acids[11] led us to consider whether they would be incorporated. We chose to investigate the chlorides **1a,b–3a,b** as potential substitutes for (*S*)-Val (**4**), (*S*)-Leu (**5**) and (2*S*,3*S*)-Ile (**6**), respectively, based on the similar van der Waals radii of chloro (1.75 Å)[12] and methyl (2.0 Å)[13] groups. Chlorine substitution is conceptually attractive because it involves replacing non-reactive amino acid side chains with functionalised derivatives. It was known already that the chloride **1a** is incorporated into proteins[14] and acts as an antagonist of (*S*)-Val (**4**) to interfere with haemoglobin biosynthesis,[15] but the efficiency and extent of its incorporation had not been investigated. The V_{max}/K_m value for its adenylation by Val-tRNA synthetase (ValRS) of *E. coli* ML30 is 30% of that of (*S*)-Val (**4**),[16] but this is only one step towards protein synthesis. (2*S*,3*R*)-Thr[17] and (*S*)-Abu are also adenylated by the ValRS but they do not even survive transfer to the related Val tRNA in the following step.[18] Even less information was available about the chloride **1b**. Its V_{max}/K_m value for adenylation by the ValRS is 1.5% of that of (*S*)-Val (**4**)[16] and it is 20% as active as the diastereomer **1a** as a Val antagonist against haemoglobin biosynthesis.[15] None of the chlorides **2a,b–3a,b** had been studied in this context, although the leucine analogue **2b** had been found to have antibacterial activity that is reversed by (*S*)-Leu (**5**).[19]



We now report that while cell-free protein synthesis of *E. coli* peptidyl-Pro *cis-trans* isomerase B (PpiB) shows no indication of incorporation of either of the Ile analogues **3a,b**, the other chlorides **1a,b–2a,b** are processed. When (*S*)-Leu (**5**) is replaced with the chlorides **2a,b** in the protein production system, PpiB is still produced as a full length, soluble protein. Similarly, each of the chlorides **1a,b** is an effective substitute for (*S*)-Val (**4**), producing soluble PpiB, at concentrations as high as those formed from (*S*)-Val (**4**). Depending on the experimental conditions, the average levels of incorporation are well above 90% and around 50% for the chlorides **1a,b**, respectively, and PpiB is formed that has up to 30% of the protein in which every one of the 16 residues of (*S*)-Val (**4**) is replaced by the chloride **1a**. Furthermore, the catalytic activity of that protein sample is very similar to that of native PpiB. In a competitive experiment the chloride **1a** is incorporated

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‡ Electronic supplementary information (ESI) available: Synthetic procedures; cell-free protein synthesis; hydrolysis of chlorides **1a,b**, **2a,b** and **3a,b**; ESI mass spectrometry of purified proteins; activity assay for PpiB; IleRS activity assay. See DOI: 10.1039/c0cc02879g

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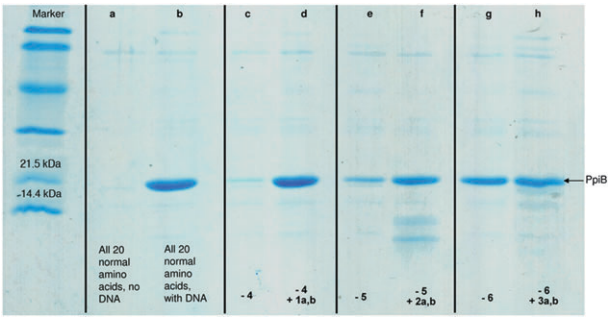

Fig. 1 SDS-PAGE gel of synthesised His_6-PpiB with: (a) no DNA; (b) DNA; (c) no (*S*)-Val (**4**); (d) no (*S*)-Val (**4**), but with the chlorides **1a,b**; (e) no (*S*)-Leu (**5**); (f) no (*S*)-Leu (**5**), but with the chlorides **2a,b**; (g) no (2*S*,3*S*)-Ile (**6**); and (h) no (2*S*,3*S*)-Ile (**6**), but with the chlorides **3a,b**.

into PpiB with an overall efficiency of ~8% of that of (*S*)-Val (**4**).

Cell-free synthesis of PpiB with an *N*-terminal His_6 tag was carried out with an *E. coli* [BL21(DE3)star] S30 extract essentially as described previously.[7,9,20] The native protein was prepared using each of the 20 normal amino acids. Incorporation of other amino acids was investigated through their substitution for one of the 20. As reported previously,[11] ~1 : 1 mixtures of the diastereomeric chlorides **1a,b–3a,b** were prepared by chlorination of (*S*)-Abu, (*S*)-Nva and (*S*)-Val (**4**), respectively. Initial examination for incorporation into PpiB involved the use of the crude diastereomeric mixtures, which also contained the corresponding starting materials and other regioisomeric chlorination products. In this form the chlorides **1a,b–3a,b** were examined at a concentration of 2 mM for each diastereomer, as substitutes for 1 mM (*S*)-Val (**4**), (*S*)-Leu (**5**) and (2*S*,3*S*)-Ile (**6**), respectively.

The proteins produced in these experiments were purified by metal-ion affinity chromatography and then analysed by 20% SDS-PAGE, as shown in Fig. 1. Mass spectral analysis of the PpiB showed that only native protein is obtained using the 20 normal amino acids, as well as from the control experiments when either (*S*)-Val (**4**), (*S*)-Leu (**5**) or (2*S*,3*S*)-Ile (**6**) is not added, and when the mixture of the chlorides **3a,b** is added instead of (2*S*,3*S*)-Ile (**6**). The production of native protein without added (*S*)-Val (**4**), (*S*)-Leu (**5**) or (2*S*,3*S*)-Ile (**6**) is attributable to the background concentrations of these amino acids, that were shown through separate HPLC analyses to increase from negligible to around 0.01 mM, in each case, during the course of the experiments. This could be the result of amino acid synthesis or release through peptide degradation. The mass spectral evidence of lack of misincorporation of either of the chlorides **3a,b** as a (2*S*,3*S*)-Ile (**6**) substitute is consistent with analysis of the gel, which shows that their addition to the medium has negligible effect on the amount of PpiB produced. On the other hand, mass spectra of PpiB produced in the presence of the chlorides **1a,b–2a,b** showed their incorporation. For the Leu analogues **2a,b**, the average level of substitution is around 90%, with around 60% of the protein having all 5 of the (*S*)-Leu (**5**) residues replaced (see ESI). The gel also shows evidence of some smaller proteins being formed in this case, that are likely to be truncated forms of PpiB, since they were not removed by ion affinity chromatography. As discussed in more detail below, high levels of substitution are observed by mass spectrometry with the Val analogues **1a,b**. These data are also consistent with the gel, which shows that addition of the chlorides **1a,b–2a,b** in the absence of (*S*)-Val (**4**) and (*S*)-Leu (**5**), respectively, increases the level of synthesis of soluble PpiB.

The chlorides **2a,b** hydrolyse to the corresponding alcohols/lactones, with half-lives of around 2.0 and 2.6 h at 37 °C under the conditions required for protein synthesis, whereas the chlorides **1a,b** and **3a,b** are stable, showing less than 5% decomposition after 6 h. It seems therefore that the misincorporation of the Val and Leu substitutes **1a,b** and **2a,b** but not the Ile analogues **3a,b** is not related to their stability, but is a true reflection of the relative extents to which they are activated and incorporated. The chlorides **1a,b** and **2a,b** are incorporated into PpiB so they must be suitable substitutes for (*S*)-Val (**4**) and (*S*)-Leu (**5**) at each and every step of protein synthesis. Incubation of the chlorides **3a,b** (2 mM) with IleRS (1.5 μM) resulted in the production of around 100% more AMP (210 μM) than when the same concentration of (2*S*,3*S*)-Ile (**6**) was used, indicating that at least one of the chlorides **3a,b** is efficiently adenylated by the synthetase. The lack of incorporation of either of the chlorides **3a,b** into PpiB must therefore be due to selectivity post adenylation.[21] Apparently, the editing process distinguishes between the $ClCH_2$ substituent of the primary chlorides **3a,b** and the $MeCH_2$ group of (2*S*,3*S*)-Ile (**6**), but not between the ClCHMe group of the secondary chlorides **1a,b** and **2a,b** and the MeCHMe group of (*S*)-Val (**4**) and (*S*)-Leu (**5**).

The chlorides **1a,b–2a,b** are two diastereomeric pairs. It was impractical to separate the Leu analogues **2a,b**, due to their instability, but the diastereomeric Val analogues **1a,b** were prepared and studied separately. Some key mass spectral data for native PpiB and the protein that formed using each of the chlorides **1a,b** at concentrations of 2, 0.2 and 0.02 mM, instead of adding 1 mM (*S*)-Val (**4**), are shown in Fig. 2, S2 and S3. The corresponding mass spectrum of PpiB prepared in a competitive experiment using the chloride **1a** (2.0 mM) and (*S*)-Val (**4**) (0.5 mM) is also illustrated in Fig. 2. The results show that both of the chlorides **1a,b** are effective substitutes

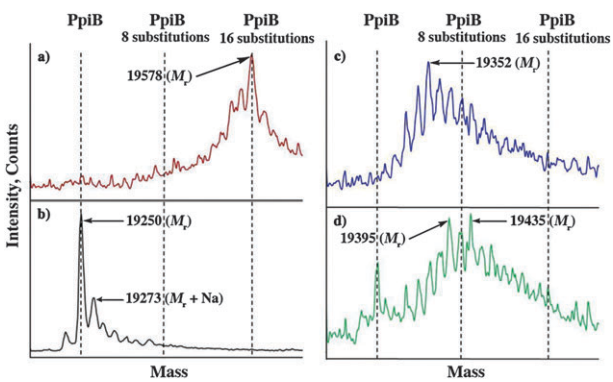

Fig. 2 Mass spectra of: (a) PpiB produced using the chloride **1a** (2 mM) instead of (*S*)-Val (**4**); (b) native PpiB; (c) PpiB produced using the chloride **1a** (2 mM) and (*S*)-Val (**4**) (0.5 mM); and (d) PpiB produced using the chloride **1b** (2 mM) instead of (*S*)-Val (**4**).

for (*S*)-Val (**4**), producing soluble PpiB at concentrations similar to those formed when (*S*)-Val (**4**) is added. Native PpiB contains 16 (*S*)-Val (**4**) residues, and the dominant peaks in the spectra of protein obtained using 2, 0.2 and 0.02 mM of the chloride **1a**, correlate with the replacement of all 16, 13–14, and 9 of these, respectively. The corresponding values for the chloride **1b** are 7–9, 5–7, and 3–5. At 2 mM concentration, the chlorides **1a,b** show average levels of incorporation of well above 90% and around 50%, respectively. The PpiB produced in the former case is around 30% comprised of protein in which every one of its 16 residues of (*S*)-Val (**4**) is replaced by the chloride **1a**.

The chloride **1b** is incorporated less efficiently than the diastereomer **1a**. In this context it is noteworthy that (2*S*,3*R*)-Thr is adenylated by the ValRS of *E. coli* ML30, but (2*S*,3*S*)-*allo*-Thr is not.[16] In this first step of incorporation, an OH group is accommodated in place of one Me substituent of (*S*)-Val (**4**) but not the other. The pattern of incorporation of the chlorides **1a,b** parallels that for adenylation of (2*S*,3*R*)-Thr and (2*S*,3*S*)-*allo*-Thr, with the Cl being preferentially accommodated in place of the Me that may be substituted for OH. The competitive experiment using the chloride **1a** (2.0 mM) and (*S*)-Val (**4**) (0.5 mM) shows an average degree of substitution of around 30% (5 of a possible 16, Fig. 2c), indicating that the overall efficiency of incorporation of the chloride **1a** is ~8% (2.0/0.5 mM × 30%) that of (*S*)-Val (**4**). In one regard this value is not surprising as it is not too dissimilar to the relative efficiency of adenylation by ValRS (30%),[16] but it is nevertheless remarkable because in direct contrast to the situation with (2*S*,3*R*)-Thr and (*S*)-Abu for example, the chloride **1a** survives editing post-adenylation. Apparently the proof-reading sieve of ValRS[18] accommodates the HOCHMe and CH_2Me side chains of (2*S*,3*R*)-Thr and (*S*)-Abu, respectively, but excludes the MeCHMe and ClCHMe groups of (*S*)-Val (**4**) and the chloride **1a**.

In conclusion, the chlorides **1a,b–2a,b** are efficiently incorporated into PpiB as substitutes for (*S*)-Val (**4**) and (*S*)-Leu (**5**), respectively, but the chlorides **3a,b** do not substitute for (2*S*,3*S*)-Ile (**6**). In preliminary studies we have also observed a virtually identical pattern of incorporation into ubiquitin. These results are of intrinsic interest, especially since protein with high levels of incorporation of chlorinated amino acids seems to be soluble and natively folded, and they also provide a way to obtain novel proteins that may have interesting properties. Conversely, it is remarkable in this regard, that PpiB formed using the chloride **1a** (2 mM), and incorporating more than 90% of that amino acid residue in place of (*S*)-Val (**4**), catalyses the interconversion of the *cis*- and *trans*-amide isomers of *N*-succinyl-(*S*)-Ala-(*S*)-Ala-(*S*)-Pro-(*S*)-Phe-4-nitroanilide with an activity similar to that of native PpiB. The K_M and k_{cat} values of the native PpiB were determined to be 380 ± 25 μM and 1940 ± 60 s^{-1}, respectively, while the corresponding values for the chlorinated protein are 590 ± 50 μM and 1110 ± 50 s^{-1}, a less than two-fold difference in each (see ESI).

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