View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

ELSEVIER
FEBS Letters 343 (1994) 70–74
FEBS 13900
FEBS LETTERS

Structural and enzymological analysis of the interaction of isolated domains of cytochrome *P*-450 BM3

Andrew W. Munro[a,]*, J. Gordon Lindsay[a], John R. Coggins[a], Sharon M. Kelly[b], Nicholas C. Price[b]

[a]*Department of Biochemistry, University of Glasgow, Glasgow, G12 8QQ, UK*
[b]*Department of Biological and Molecular Sciences, University of Stirling, Stirling, FK9 4LA, UK*

Received 7 March 1994

Abstract

The interactions of the individually expressed haem- and flavin-containing domains of cytochrome *P*-450 BM3 have been analysed by enzymological and spectroscopic techniques. Electron transfer between the isolated domains occurs at a much lower rate than that occurring in the intact flavocytochrome. CD spectroscopic studies indicate that the linkage of the domains in intact *P*-450 BM3 creates haem and amino acid environments suitable for efficient electron transfer from its flavin domain.

Key words: Cytochrome *P*-450 BM3; Domain interaction; Circular dichroism; Electron transfer; Haem environment

1. Introduction

The cytochrome *P*-450 BM3 system from *Bacillus megaterium* provides a unique bacterial model for mammalian *P*-450 systems, such as those involved in drug and xenobiotic metabolism [1–3]. It is the only characterised prokaryotic class II *P*-450 utilising an FAD/FMN containing NADPH-*P*-450 reductase as an electron transfer partner [4]. Other prokaryotic (class I) systems operate a three protein-component electron transfer chain with electrons flowing from NAD(P)H onto an FAD-containing redoxin reductase, through an iron-sulphur redoxin and onto the *P*-450 (e.g. [5]). In addition, *P*-450 BM3 has its redox partners fused in a single polypeptide chain, with the haem-containing *P*-450 'domain' being N-terminal and the flavincontaining reductase C-terminal [4,6]. The only structurally analogous *P*-450 yet characterised is the cell signalling enzyme nitric oxide (NO) synthase [7].

Like all prokaryotic *P*-450s, *P*-450 BM3 is a soluble enzyme. This has enabled relatively simple purification of the polypeptide [4]. The gene has been cloned and expressed in *E. coli* [8,9], as have the sub-genes encoding the constituent domains [9–11]. The X-ray structure of the *P*-450 domain has recently been determined [12], providing a long-awaited atomic model for the class II *P*-450s.

The ability to isolate fully active intact *P*-450 BM3 and its haem- and flavin-containing domains opens the way for detailed analysis of the separate active sites, as well as the mechanism of electron transfer between the redox centres. This paper reports the results of experiments in which the interaction between the two domains of *P*-450 BM3 is studied using a combination of kinetic and spectroscopic techniques. We demonstrate here, for the first time, that the linking of the domains in the intact polypeptide leads to alterations in the haem and aromatic amino acid environments which presumably provide the correct structural basis for efficient transfer of electrons between the redox centres.

2. Materials and methods

2.1. E. coli *strains, plasmid and bacteriophage vectors*

E. coli strains TG1 (*supE*, *hsd*Δ5, *thi*, Δ(*lac-pro*AB), F′ [*tra*D36, *pro*AB$^+$, *lac*I^q, *lac*ZΔM15]) [13] and XL-1 Blue (*sup*E44, *hsd*R17, *rec*A1, *gyr*A46, *thi*, *rel*A1, *lac*$^-$, F′ [*pro*AB$^+$, *lac*I^q, *lac*ZΔM15, Tn10(tet^r)]) [14] were used for plasmid propagation and the overexpression of the gene encoding cytochrome *P*-450 BM3 (*CYP*102) and the PCR-generated sub-genes encoding its constituent reductase and haem 'domains'. Construction of plasmids used for expression of *P*-450 BM3 and its constituent domains in *E. coli* has been described previously [9]. Briefly, pJM23 consists of a 5-kb segment of *B. megaterium* chromosomal DNA (containing the *CYP*102 gene) cloned as *Xba*I–*Eco*RI restriction fragment into vector pUC119 [15] and expression is from the *Bacillus* promoter. Plasmid pJM27 was used for expression of the flavoprotein reductase domain of *P*-450 BM3 (initiating methionine and residues 473 to end) and consists of an approximately 1.8 kb PCR fragment of

*Corresponding author. Fax: (44) (41) 330 4620.

Abbreviations: *P*-450, cytochrome *P*-450 linked monooxygenase; IPTG, isopropyl-β-D-thio-galactopyranoside; MOPS, morpholinopropanesulphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; CO, carbon monoxide; NO, nitric oxide; CD, circular dichroism; EPR, electron paramagnetic resonance; UV, ultra violet.

0014-5793/94/$7.00 © 1994 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(94)00285-4

*CYP*102 cut with *Bam*HI (to generate cohesive ends from restriction enzyme sites engineered at 5′ ends of PCR primers) and cloned into vector p*tac*85 under an inducible *tac* promoter [16]. Plasmid pJM20 was used for the expression of the haem-containing domain of *P*-450 BM3 (residues 1 to 472) and consists of an approximately 1.5 kb PCR fragment of *CYP*102 cut with *Eco*RI and *Bam*HI (to generate cohesive ends as with pJM27) and cloned into pUC118 [15] under the *lac* promoter. Other bacterial strains and vectors used in these studies have been described previously [9].

2.2. Molecular biology techniques

DNA manipulations, bacterial transformations and other molecular techniques were performed by standard methods [17].

2.3. Expression and purification of intact cytochrome P-450 BM3 and its constituent domains

E. coli transformants carrying plasmids encoding wild-type *P*-450 BM3 and its constituent flavoprotein reductase domain were grown overnight to high cell density in Terrific Broth plus antibiotic (ampicillin) [18]. IPTG inducer (final concentration 25 μg/ml) was added to facilitate expression from plasmids pJM27 (reductase domain) and pJM20 (haem-containing domain). Intact *P*-450 BM3 was expressed from plasmid pJM23 under the control of its own promoter and without induction, as previously described [9]. Expression levels of intact *P*-450 BM3 were seen to increase significantly in cultures of pJM23 transformants grown to stationary phase, as has been previously reported [19].

Approximately 30 g of wet cell pellets were the starting points for polypeptide purification. Following cell rupture by French pressure, polypeptides were purified to homogeneity by successive steps of ammonium sulphate precipitation, ion exchange chromatography on DEAE-Sephacel and either affinity chromatography on 2′,5′-ADP-Sepharose (for intact *P*-450 BM3 and its reductase domain) or by affinity for Bio-Gel HTP (DNA grade) hydroxylapatite (for the haem domain of *P*-450 BM3) as previously described [9]. Purification of the polypeptides by gel filtration using Sephacryl S-300 HR was used as a final purification step, when required.

2.4. Spectroscopy, protein and enzyme assays

All UV-visible spectroscopy was performed on a Shimadzu 2100 spectrophotometer (Shimadzu corporation, Kyoto, Japan). Protein concentrations were determined using the methods of Bradford [20] and by the BCA technique [21] with BSA as standard. Cytochrome *P*-450 concentrations were estimated by the method of Omura and Sato [22] using $\varepsilon = 91\ \mathrm{mM^{-1} \cdot cm^{-1}}$ at 450 nm for the reduced plus CO adduct. NADPH-dependent fatty acid hydroxylation was measured at 30°C in 20 mM MOPS buffer (pH 7.4) containing 100 mM KCl, using $\varepsilon = 6.2\ \mathrm{M^{-1} \cdot cm^{-1}}$ at 340 nm. The buffer system contained 0.5 mM sodium laurate and 0.2 mM NADPH. Domain coupling experiments were performed under similar conditions in 20 mM MOPS buffer (pH 7.4) containing KCl at concentrations between 0 and 100 mM. Substrate-independent NADPH oxidation was assayed under the same conditions, but in the absence of sodium laurate. Cytochrome *c* reductase activity was determined using $\varepsilon = 21\ \mathrm{mM^{-1} \cdot cm^{-1}}$ at 550 nm in 20 mM MOPS (pH 7.4) containing 100 mM KCl, as previously described [9]. Rates shown are the means of at least three separate determinations, differing by less than 10%. CD spectra were recorded on a Jasco J-600 spectropolarimeter. Far-UV CD spectra were analysed for secondary structure by the CONTIN procedure [23].

2.5. Materials

Molecular biology reagents were purchased from Boehringer or United States Biochemicals. DEAE-Sephacel was purchased from Pharmacia-LKB. All other reagents and enzymes were obtained from Sigma.

3. Results

3.1. Kinetic studies on domain interactions

As shown in Table 1, the rate of substrate-dependent NADPH oxidation in the intact enzyme was approximately 1300 nmol/min/nmol for the preparation used. When the separate domains were mixed in stoichiometric proportions, the rates of NADPH oxidation were approximately 1000-fold lower. Preliminary stopped flow kinetic data indicate that haem reduction may be the rate limiting step in *P*-450 BM3's catalytic cycle, so the rates of NADPH oxidation may be regarded as approximately equivalent to those of inter-domain electron transfer. Higher overall rates were achieved when larger quantities of domains were mixed (expressed as 'nmol/min/nmol *P*-450 domain'). However, the main reason for this is that the reductase domain of *P*-450 BM3 acts as a slow NADPH oxidase in the absence of added electron acceptor [24]. The 'fatty acid-dependent' and '*P*-450-dependent' rates in Table 1 have been corrected for this. In addition, we find that electron transfer between the separated domains is more efficient in buffer solutions containing at least approx. 20 mM KCl than it is in the absence of salt. This is also found to be the case when electron transfer from the reductase domain to cytochrome c is measured. Our findings that the rate of electron transfer between the separated domains is very low are in agreement with the results of Li et al. [10] and Narhi and Fulco [6] who used domains constructed genetically and generated by trypsinolysis of intact enzyme, respectively.

Table 1
Rates of NADPH oxidation catalysed by *P*-450 BM3, its constituent domains and by mixtures of its constituent domains are shown

Enzyme	Overall rate	Substrate-independent rate	Fatty acid-dependent rate	*P*-450-dependent rate
P-450 BM3	1280	1.1	1279	1279
R domain	0.7	0.7	n.a.	n.a.
P domain	< 0.01	n.a.	n.a.	n.a.
R + P	1.1	0.7	0.4	0.4
R + 2P	1.3	0.7	0.6	0.3
R + 3P	1.7	0.7	1.0	0.3
R + 10P	3.4	0.7	2.7	0.3
0.5R + 3P	2.0	0.7	1.35	0.2
2R + 3P	1.4	0.7	0.7	0.5
3R + 3P	1.3	0.7	0.6	0.6

Overall rates are expressed as 'mol/min/mol enzyme' where one enzyme has been used, and 'mol/min/mol' of reductase domain in the case of the mixtures. The substrate-independent rate in the mixtures is shown as 'mol/min/mol reductase domain' and is substracted from the overall rate to give a fatty acid dependent rate. This is then converted into the fatty acid-dependent rate expressed as 'mol/min/mol *P*-450 domain' (*P*-450-dependent rate) through multiplication by the appropriate factor (factor = quantity reductase domain used/quantity *P*-450 domain used). In the case of the domains and mixtures thereof, 'R' and 'P' represent the reductase and *P*-450 domains used at a final concentration of 0.75 mM. Similarly, '0.5R' is reductase domain at 0.375 mM; '2P' is *P*-450 domain at 1.5 mM and so on. Results shown are averages of triplicate readings, differing by less than 3%. Assays were performed as described in section 2. (n.a., not appropriate to express rates in units used).

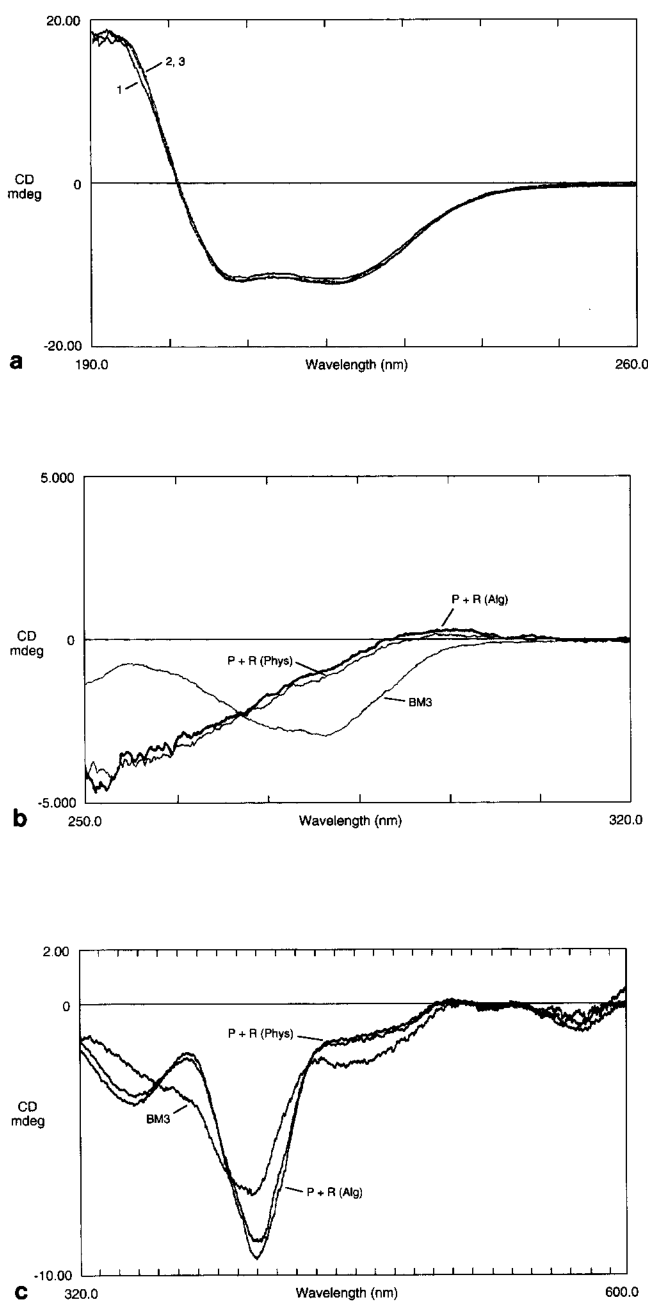

Fig. 1. (a) The far-UV CD spectra of (1) intact cytochrome *P*-450 BM3 (3.15 μM), (2) the algebraic addition of the individual spectra of its independently purified *P*-450 and reductase domains (at the same concentration), and (3) the mixture of the domains (at the same concentration). (b) (c) The near-UV and visible CD spectra, respectively, of the corresponding protein solutions (all at concentrations of 9.45 μM). The pathlengths in a, b and c were 0.02 cm, 0.5 cm and 0.5 cm, respectively.

3.2. Mutual affinity of domains

The interactions between the isolated domains were investigated in two ways. Firstly, the concentration of the reductase domain was varied at a fixed concentration of the *P*-450 domain and the data analysed as a Hanes plot ([S]/v versus [S]). From this, the K_m of the reductase domain (which can be regarded as a measure of the mutual affinity of the domains) could be estimated at 0.72 mM. Secondly, the ability of the reductase domain immobilised on 2,′5′-ADP Sepharose to bind the *P*-450 domain was examined at variety of ionic strengths. Even under optimal conditions (100 mM KCl), only very slight interaction was observed, as indicated by a slight retardation of the elution of added *P*-450 domain from the column. This weak interaction would be consistent with the high K_m value obtained in the kinetic studies.

3.3. Circular dichroism studies on P-450 *BM3 and its domains*

CD spectra of the isolated domains and the intact enzyme were recorded in the far-UV, near-UV and visible regions. As shown in Fig. 1a, the far-UV spectra of the isolated domains could be added together to generate a spectrum indistinguishable from that of the intact enzyme. This latter spectrum is also identical with that of the stoichiometric mixture of domains. It is thus clear that the isolated domains retain their secondary structure. Applying the CONTIN procedure [23] to these spectra indicated that the contents of α-helix and β-sheet in each case were 34% and 30%, respectively.

However, in the near-UV and visible regions (Fig. 1b and c), there are pronounced differences between the added spectra of the isolated domains and the spectrum of intact enzyme. The spectrum obtained by addition of the spectra of the isolated domains was very similar to that of the stoichiometric mixture. This would be consistent with the low degree of mutual affinity referred to above. Thus, for the first time, we have been able to show that the environments of the aromatic amino acids and haem in the intact enzyme are different from those in the isolated domains.

4. Discussion

It is well recognised that polypeptide chains containing about 200 amino acids have a tendency to form independently folding units, or domains [25]. Such multiple domains can create appropriate environments for catalytic activity and can provide the means by which different catalytic activities may be linked together efficiently. The *P*-450 BM3 enzyme offers an excellent system for the study of domains and their interactions. The two major domains of the enzyme each possess characteristic catalytic and spectroscopic properties and can be expressed independently of each other in high yield. It should also be noted that the reductase domain itself belongs to a small, but evolutionarily related, family of enzymes formed from the fusion of two smaller flavin-containing 'sub-domains' [26].

The rate of electron transfer between the isolated domains is very much slower than that between the redox centres of the *P*-450 BM3 enzyme (Table 1). While this is in agreement with the findings of Li et al. [10] and

Tyr-Glu-Leu-Asp-Ile-Lys-Glu-Thr-Leu-Thr-**Lys**-Pro-Glu-
432 440

Gly-Phe-Val-Val-Lys-Ala-**Lys**-Ser-Lys-**Lys**-Ile-Pro-Leu-
450

Gly-Gly-Ile-Pro-Ser-Pro-Ser-Thr-Glu-Gln-Ser-Ala-**Lys**-**Lys**-
460 470

Val-Arg-Lys-Lys-Ala-Glu-Asn-Ala-His-Asn-Thr-Pro-Leu-
472 480

Leu-Val-Leu-Tyr-Gly-Ser-Asn-Met-Gly-Thr-Ala-Glu-Gly.
490 496

Fig. 2. The amino acid sequence of cytochrome *P*-450 BM3 is shown at the boundary region of the *P*-450 and reductase domains (residues 432–496). The arginine residue (Arg^{472}) recognised as preceding the favoured inter-domain trypsin site is in outlined type. Residues preceding other potential trypsinolysis sites are in bold type. 'Desirable' linker residues [30] are underlined. Based on alignments with other known cytochromes *P*-450 and comparisons with the lengths of other known linkers, it is likely that the *P*-450 domain terminates at approx. Ser^{462} and that the entire linker is contained within the next 10–15 residues.

Narhi and Fulco [6], it has been reported by Boddupalli et al. [27] that the isolated domains can couple catalytically to an extent comparable with that in the intact enzyme. However, this conclusion must be regarded with caution in view of the facts that the NADPH oxidase activity was not taken into account and that saturation kinetic behaviour was not convincingly demonstrated.

In view of the relatively weak mutual affinity of the domains shown in our kinetic and binding experiments, it would appear that the linker region in the intact enzyme plays an important role in maintaining the two major domains in close proximity so as to enhance the rate of electron transfer. Inspection of the amino acid sequence about the linker region (Fig. 2) shows that it is rich in charged residues (e.g. 9 Lys, 1 Arg, 6 Glu), but also contains 4 Pro. This sequence does not resemble any of the other well characterised linker regions in proteins; such as the 'Q' linkers located at the boundaries of domains in bacterial regulatory and sensory transduction proteins [28], the linkers characterised by Ala-Pro repeats which are common in decarboxylases and dehydrogenases [29] or the 'LKTPGRED' linkers from bacterial phosphotransferase system enzymes [30]. However, it does contain a preponderance of amino acids considered favourable as constituents of inter-domain linkers [31]. The exact position of the linker region is not well defined. However, multiple alignment of the *P*-450 BM3 amino acid sequence with those of sixty other known *P*-450 primary sequences shows that the vast majority (45) terminate at amino acids within two residues of that amino acid aligning with Ser^{462} of *P*-450 BM3, and that all of the others terminate at or before that amino acid aligning with Arg^{472}. With this in mind, and given the fact that most characterised linker units are around four to twelve amino acids long [31], it may be the case that residues 460 to approx. 475 encompass the entire linker region of *P*-450 BM3.

From the far-UV CD spectra (Fig. 1a), it is evident that the joining of the major domains in the intact enzyme is not accompanied by any significant change in secondary structure. Previous EPR studies indicated the haem ligation state in intact *P*-450 BM3 to be unaltered from that in the isolated *P*-450 domain [9]. However, the interaction between the domains clearly involves localised structural alterations as manifested by the differences in the aromatic amino acid and haem environments detected in the near-UV and visible regions (Fig. 1b and c). Although we are unable, at present, to define these structural alterations, it is reasonable to assume that they are required for the efficient transfer of electrons within the intact enzyme. The observed transfer rate for this enzyme is amongst the very highest yet reported for any *P*-450 system [4]. A precise definition of the structural alterations involved in domain interaction will await X-ray crystallographic analysis of the intact *P*-450 BM3.

Acknowledgements: We wish to thank the SERC for financial support.

References

[1] Guengerich, F.P. (1991) J. Biol. Chem. 266, 10019–10022.
[2] Black, S.D. and Coon, M.J. (1987) Adv. Enzymol. Relat. Areas Mol. Biol. 60, 35–87.
[3] Porter, T.D. and Coon, M.J. (1991) J. Biol. Chem. 266, 13469–13472.
[4] Narhi, L.O. and Fulco, A.J. (1986) J. Biol. Chem. 261, 7160–7169.
[5] Katagiri, M., Ganguli, B.N. and Gunsalus, I.C. (1968) J. Biol. Chem. 243, 3543–3546.
[6] Narhi, L.O. and Fulco, A.J. (1987) J. Biol. Chem. 262, 6683–6690.
[7] Bredt, D.S., Hwang, P.M., Glatt, C.E., Lowenstein, C., Reed, R.R. and Snyder, S.S. (1991) Nature 351, 714–718.
[8] Wen, L.-P. and Fulco, A.J. (1987) J. Biol. Chem. 262, 6676–6682.
[9] Miles, J.S., Munro, A.W., Rospendowski, B.N., Smith, W.E., McKnight, J. and Thomson, A.J. (1992) Biochem. J. 288, 503–509.
[10] Li, H., Darwish, K. and Poulos, T.L. (1991) J. Biol. Chem. 266, 11909–11914.
[11] Oster, T., Boddupalli, S.S. and Peterson, J.A. (1991) J. Biol. Chem. 266, 22718–22725.
[12] Ravichandran, K.G., Boddupalli, S.S., Hasemann, C.A., Peterson, J.A. and Deisenhofer, J.A. (1993) Science 261, 731–736.
[13] Gibson, T.J. (1984) Ph.D. thesis, University of Cambridge, England.
[14] Bullock, W.O., Fernandez, J.M. and Short, J.M. (1987) Biotechniques 5, 376–379.
[15] Vieira, J. and Messing, J. (1987) Methods Enzymol. 153, 3–11.
[16] Marsh, P. (1986) Nucleic Acids Res. 14, 3603.

[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A Laboratory Manual (2nd edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Tartof, K.D. and Hobbs, C.A. (1987) Bethesda Res. Lab. Focus 9, 12.
[19] Narhi, L.O. and Fulco, A.J. (1982) J. Biol. Chem. 257, 2147–2150.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[22] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385.
[23] Provencher, S.W. and Glöckner, J. (1981) Biochemistry 20, 33–37.
[24] Munro, A.W., Lindsay, J.G. and Coggins, J.R. (1993) Biochem. Soc. Trans. 21, 412S.
[25] Hardie, D.G. and Coggins, J.R. (1986) in: Multidomain Proteins: Structure and Evolution (Hardie, D.G. and Coggins, J.R., Eds.) pp. 333–344, Elsevier, Amsterdam.
[26] Porter, T.D. (1991) Trends Biochem. Sci. 16, 154–158.
[27] Boddupalli, S.S., Oster, T., Estabrook, R.W. and Peterson, J.A. (1992) J. Biol. Chem. 267, 10375–10380.
[28] Wootton, J.C. and Drummond, M.H. (1989) Prot. Eng. 2, 535–543.
[29] Perham, R.N. (1991) Biochemistry 30, 8501–8512.
[30] Schunk, T., Rhiel, E., de Meyer, R., Buhr, A., Hummel, U., Wehrli, C., Flükiger, K. and Erni, B. (1992) in: Molecular Mechanisms of Transport (Quagliariello, E. and Palmieri, F., Eds.) pp. 87–95, Elsevier, Amsterdam.
[31] Argos, P. (1990) J. Mol. Biol. 211, 943–958.