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Site-directed mutagenesis of the proton-pumping pyridine nucleotide transhydrogenase of *Escherichia coli*

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Abstract

The pyridine nucleotide transhydrogenase of *Escherichia coli* catalyzes the reversible transfer of hydride ion equivalents between NAD^+ and $NADP^+$ coupled to the translocation of protons across the cytoplasmic membrane. It is composed of two subunits (α, β) organized as an $\alpha_2\beta_2$ tetramer. This brief review describes the use of site-directed mutagenesis to investigate the structure, mechanism and assembly of the transhydrogenase. This technique has located the binding sites for NAD(H) and NADP(H) in the α and β subunits, respectively. Mutagenesis has shown that the cysteine residues of the enzyme are not essential for its function, and that inhibition of the enzyme by sulfhydryl-specific reagents must be due to perturbation of the three-dimensional structure. The sites of reaction of the inhibitors *N,N'*-dicyclohexylcarbodiimide and *N*-(1-pyrene)maleimide have been located. Selective mutation and insertion of cysteine residues followed by cupric *o*-phenanthrolinate-induced disulfide crosslinking has defined a region of interaction between the α subunits in the holoenzyme. Determination of the accessibility of selectively inserted cysteine residues has been used to determine the folding pattern of the transmembrane helices of the β subunit. Site-directed mutagenesis of the transmembrane domain of the β subunit has permitted the identification of histidine, aspartic acid and asparagine residues which are part of the proton-pumping pathway of the transhydrogenase. Site-directed mutagenesis and amino acid deletions have shown that the six carboxy terminal residues of the α subunit and the two carboxy terminal residues of the β subunit are necessary for correct assembly of the transhydrogenase in the cytoplasmic membrane. © 1998 Elsevier Science B.V.

Keywords: Transhydrogenase; Proton pumping; Proton pathway; Pyridine nucleotide-binding site; Site-directed mutagenesis; Inhibition site

1. Introduction

Pyridine nucleotide transhydrogenase is a proton pump which catalyzes the reversible reaction

$$H^+_{out} + NADP^+ + NADH \rightleftharpoons NADPH + NAD^+ + H^+_{in}$$

where reduction of $NADP^+$ by NADH is linked to an inward translocation of protons from the periplasm (in bacteria) or cytosol (mammalian cells) into the cytosol or mitochondrial matrix, respectively. In the presence of an electrochemical proton gradient generated by another proton pump, the apparent equilibrium of the reaction is shifted strongly towards formation of NADPH and NAD^+, and the rate of reduction of $NADP^+$ by NADH is increased 10-fold. Proton translocation coupled to the reduction of

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NAD^+ by NADPH is driven by the difference in the binding energies of the reactants (NADPH and NAD^+) and the products ($NADP^+$ and NADH). Since binding of NADPH and $NADP^+$, but not of NADH and NAD^+, alter the conformation of the enzyme, it is likely that the difference in binding energies of NADPH and $NADP^+$ is the principal driving force for proton pumping [1–3]. The pyridine nucleotide transhydrogenase of *Escherichia coli* is composed of two subunits, α (510 residues) and β (462 residues), organized as an $\alpha_2\beta_2$ tetramer [5–7]. The relative simplicity of this structure makes the enzyme an excellent system in which to study the mechanism of proton pumping. Three domains are recognized in transhydrogenases [2,7,8]. Domains I and III are extramembrane domains carrying the NAD(H)- and NADP(H)-binding sites, respectively. Domain II is inserted in the bacterial cell or mitochondrial inner membrane. The properties of transhydrogenases have been reviewed recently by Hatefi and Yamaguchi [2] and Olausson et al. [7].

Site-directed mutagenesis allows specific replacement of selected amino acid residues by other amino acids. This permits investigation of the role of these amino acids in a protein. As outlined in this brief review, we have used this technique to study the structure, mechanism and assembly of the pyridine nucleotide transhydrogenase of *E. coli*.

2. Mutagenesis of the pyridine nucleotide-binding sites

The amino acid sequence of the transhydrogenase revealed a putative pyridine nucleotide-binding site on the α and the β subunits [4,5,9]. The GXGXXG motif beginning at residue αGly^{172} is found at the first β–α turn of the βαβ-fold typical of dinucleotide-binding enzymes [10] (Fig. 1). The pyrophosphate bridge of the dinucleotide binds at this turn. Conserved glycine and aspartate residues at positions 188 and 195 are typical of NAD(H)-binding sites (Fig. 1), and indicate that the NAD(H)-binding site is on the α subunit. The sequence motif GXGXXV beginning at residue βGly^{314} is more typical of NADP(H)-binding sites [9,11]. The presence of conserved residues (βAla^{327}, βGly^{337}, βHis^{345} and βArg^{350}) shared with other NADP(H)-binding enzymes [9,11] (Fig. 1) support the assignment of the NADP(H)-binding site to the β subunit of the transhydrogenase.

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TH (NAD site)      GAGVAGLAAIGAANSLG-A-IVRAFDTRPEVKEQVQSM
                  174 176          188      195

Alco DH (Horse)    GLGGVGLSVIMGCKAAGAA-RIIGVDINKDKFAKAKEV
Glyc DH (Pig)      GFGRIGRLVTRAAFNSGKVDIVAINDPFIDLHYMVYMF
Lipo DH (Human)    GAGVIGVELGSVWQRLG-AD-VTAVEFLGHVGGVGIDM
Lact DH (Pig)      GVGAVGMACAISILGKELADEIALVDVMEDKLKGEMMD


TH (NADP site)     GYGMAVAQAQYPVAEITEKLRARGINVRFGIHPVAG-R
                  314   319      327        337     345   350

GSH red (E.coli)   GAGYIAVEL----AGVINGL---GAKTHLFVRKHAPLR
Thio red (E.coli)  GGGNTAVEE----ALYLSN----IASEVHLIHRRDGFR
Tryp red           GGGFISVEF----AGIFNAYKPVGGKVTLCYRNNPILR
Mer red(Shigella)  GSSVVALEL----AQAFARL---GAKVTILARSTLFFR

TH (Beta 274-311)  GFGTDGSSTGDDQEVGEHREITAEETAELLKNSHSVII
                  274 276
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Fig. 1. Comparison of amino acid sequences at NAD(H)- and NADP(H)-binding sites of several enzymes. Conserved amino acids are in bold letters. Alco DH, alcohol dehydrogenase; Glyc DH, glyceraldehyde-3-phosphate dehydrogenase; Lipo DH, lipoamide dehydrogenase; Lact DH, lactate dehydrogenase; GSH red, glutathione reductase; Thio red, thioredoxin reductase; Trp red, trypanothione reductase (Trypanosoma); Mer red, mercuric reductase; TH, *E. coli* pyridine nucleotide transhydrogenase.

Site-directed mutagenesis has revealed the importance of the conserved residues of the putative binding sites. Mutagenesis of the first glycine residue (αGly^{172} or βGly^{314}) in the GXGXXG(V) motifs resulted in complete loss of catalytic activity [5,12,13]. The effects of the mutation in the β subunit were particularly interesting since the NADP(H)-induced cleavage of the β subunit at βArg^{265} by trypsin was abolished [5] thus supporting the assignment of NADP(H) site to the β subunit. Mutations at αGly^{174}, βVal^{319}, βGly^{337} and βHis^{345} had less drastic effects [13,14]. Mutation of βArg^{350} abolished catalytic activity [13]. This residue probably interacts with the 2′-phosphate of the substrate NADP(H).

The covalently modifying nucleotide analog 5′-(*p*-fluorosulfonylbenzoyl)adenosine (FSBA) was used by Hatefi and co-workers [15,16] to identify residues involved in the substrate-binding sites of the mitochondrial pyridine nucleotide transhydrogenase. Two tyrosine residues, Tyr^{245} and Tyr^{1006}, were specifically modified resulting in inhibition of the enzyme. These residues are equivalent to αTyr^{226} and βTyr^{431} in the *E. coli* transhydrogenase. Mutagenesis has revealed that neither amino acid is essential for catalytic activity in the *E. coli* enzyme [17]. Inhibition by FSBA must therefore be due to the introduction of a sterically hindering group at the active site. An analogous effect was produced by covalent modification of the NAD(H) site with *N,N′*-dicyclohexylcarbodiimide (DCCD). This reagent modified αAsp^{232}, αGlu^{238} and αGlu^{240} with loss of activity (Glu^{257}, equivalent to *E. coli* αGlu^{238}, was modified in the mitochondrial enzyme [16]). Mutagenesis showed that none of these residues was essential for catalytic activity [18]. The presence of the FSBA- and DCCD-modifiable residues at the active site is clearly seen in a model of the NAD-binding site [12].

The β subunit contains a second GXGXXG motif starting at βGly^{274}. The possibility that this might be a second nucleotide-binding site on the β subunit was examined since indirect evidence had been obtained that a second site was present on the β subunit [19]. Thus, the β subunit of the transhydrogenase when bound to NAD-agarose was cleaved by trypsin only in the presence of NADP(H). This result suggested that the β subunit had binding sites both for NADP(H) and NAD. Mutagenesis of βGly^{274} and βGly^{276} had little effect on catalytic activities [13]. Thus, the GXGXXG motif at βGly^{274} is unlikely to be part of a pyridine nucleotide-binding site. This is consistent with the absence of conserved 'downstream' residues typical of a NAD(H)-binding site.

3. Mutagenesis of cysteine residues

Reaction of the *E. coli* transhydrogenase with sulfhydryl-specific reagents results in inhibition of catalytic activity [20]. It has been suggested that hydride transfer between the two pyridine nucleotide substrates involves reduction and oxidation of a pair of sulfhydryl residues sufficiently close to permit the formation of a disulfide bond [21]. Mutation of the cysteine residues has provided information on both the structure and mechanism of the enzyme [22,23].

E. coli transhydrogenase contains seven cysteine residues, five in the α subunit (αCys^{292}, αCys^{339}, αCys^{395}, αCys^{397} and αCys^{435}) and two in the β subunit (βCys^{147} and βCys^{260}). A cysteine-free enzyme was generated in which all cysteine residues had been replaced by serine or threonine residues. Since it retained catalytic activities at least 30% of those of nonmutant transhydrogenase [22], hydride transfer between the two substrates must not involve intermediary reduction/oxidation of a pair of sulfhydryl residues. It has been shown recently that there is direct transfer of hydride ion equivalents between the pyridine nucleotide substrates [24]. By using mutants in which only selected cysteine residues were retained, it was shown that the likely site of reaction of sulfhydryl reagents leading to inhibition of catalytic activity is βCys^{260} [23]. As with FSBA and DCCD, inhibition must be due to perturbation of the structure of the enzyme since mutation of βCys^{260} has little effect on enzyme activity.

Site-directed cysteine mutants were used in two further structural studies. The three-dimensional structures of relatively few membrane enzymes are known. Thus, other methods have been employed to provide structural information. Methods have been developed to take advantage of the reactivity of the thiol groups of cysteine residues in enzymes. For example, cross-linking studies had shown that α_2, $\alpha\beta$ and β_2 dimers are generated by crosslinking of the subunits of the *E. coli* transhydrogenase [6]. This,

together with radiation inactivation analysis indicates that the native enzyme is an $\alpha_2\beta_2$ tetramer. The α_2 dimers were readily generated by oxidation of sulfhydryl pairs with cupric *o*-phenanthrolinate. Mutation of αCys^{395} and αCys^{397} prevented disulfide bond formation which gave the crosslinked α_2 dimer. Thus, the two α subunits of the holoenzyme interface in the region of these two cysteine residue [23].

Hydropathy plots indicate that the amino terminal region of the β subunit to residue 260 is transmembranous [25]. Models of the topology of this region predict that eight or six transmembrane α-helices are present [25,26]. Examination of the reactivity of native cysteine residues, or of those inserted in selected regions by site-directed mutagenesis, with 3-(*N*-maleimidyl propionyl)biocytin or fluorescent sulfhydryl-specific reagents revealed a labelling pattern supportive of the eight α-helices model [27].

The reagent *N*-(1-pyrene)maleimide has been found to be a useful reagent to investigate some aspects of the structure of proteins. It forms eximers (excited-state dimers) after reaction with membrane proteins such as the Ca^{2+}-ATPase of sarcoplasmic reticulum [28] and the lactose permease of *E. coli* [29]. Eximers are formed if the pyrene ring systems are within 0.35 nm and in the correct orientation. Thus, the proximity of cysteine residues in pyrene maleimide-treated proteins can be determined by appearance of an eximer band. This approach was applied to various mutants of the *E. coli* transhydrogenase retaining one or more of the original cysteine residues present [23]. An eximer band was formed even with cysteine-free transhydrogenase. Thus, there are significant limitations in the use of this reagent to study membrane protein structure.

4. Mutagenesis of domain II

Domain II is the transmembrane domain of the transhydrogenase and must contain the pathway by which protons are pumped across the membrane. In *E. coli*, domain II is composed of the C-terminal 100 residues of the α subunit and the N-terminal 260 residues of the β subunit. This region of the α subunit is organized as four transmembrane α-helices. The β subunit region contains eight transmembrane α-helices [27]. The topology of domain II is shown in Fig. 2.

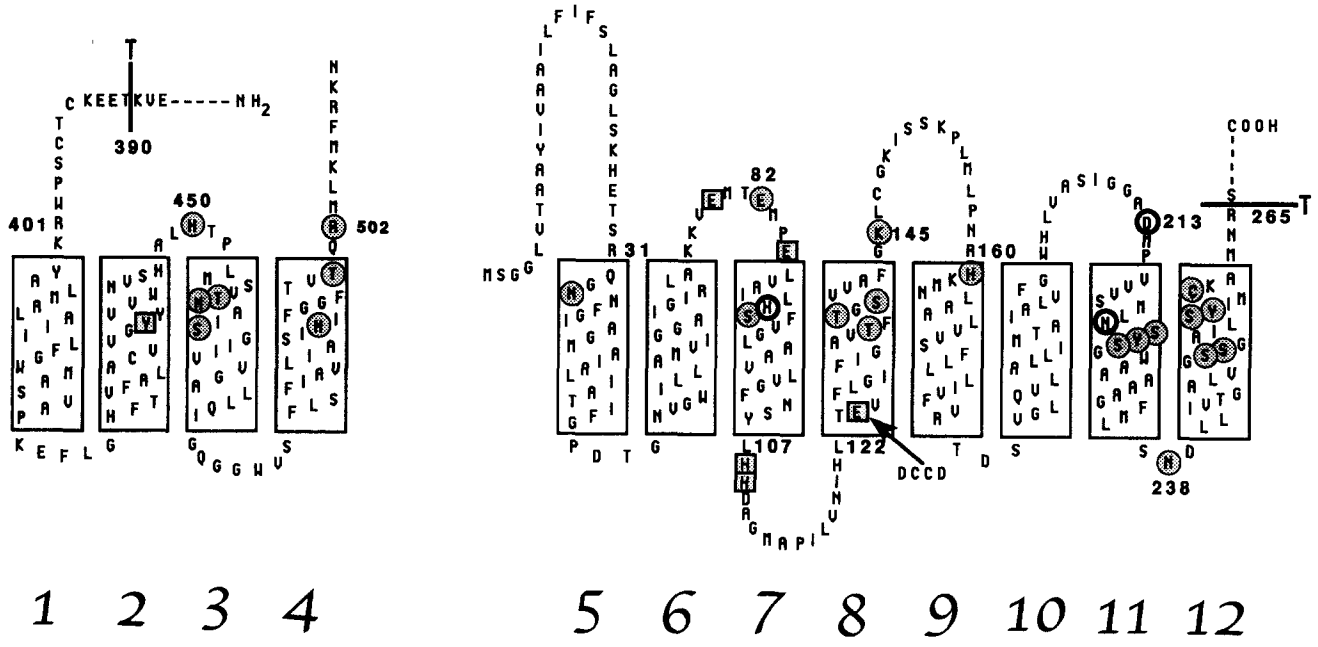

Fig. 2. Model of the topology of the transmembrane domains (domain II) of the α and β subunits of the *E. coli* transhydrogenase. Putative amino acids of the proton pathway are ringed in bold. Conserved amino acid residues which have been mutated are indicated with a stippled circle. Mutated semi- or nonconserved amino acids are shown in stippled boxes. The DCCD-modifiable βGlu^{124} is indicated.

The pathways by which protons are pumped across membranes are still uncertain although the recent crystal structures of cytochrome *c* oxidase and bacteriorhodopsin [30,31] have provided some clues. Protons are transferred most effectively through hydrogen bonds, particularly in a nonpolar environment such as the transmembrane regions of proteins. Thus, a hydrogen-bonded network acting as a relay system to translocate protons from one side of the membrane to the other would be expected. Such a system also needs a mechanism by which a conformational change mediated by a redox change, ligand binding or photoexcitation can induce changes in the pK_a of ionizable amino acids. Conserved ionizable amino acid residues were mutated to identify residues involved in the proton pathway through the membrane domain (domain II) of the pyridine nucleotide transhydrogenase of *E. coli*. Mutation of αHis^{450}, αArg^{502}, βGlu^{82}, βLys^{145}, βHis^{160} and βAsp^{213} produced relatively small changes in hydride transfer and proton pumping activities [26]. By contrast, most mutants of βHis^{91} were more severely affected with the exception of $\beta H^{91}N$ [26,32] (Table 1). This mutant was particularly interesting since it showed an 'uncoupled phenotype', that is, little loss of catalytic activity while proton translocation was abolished. (The low proton translocation activity seen in Table 1 is due to the activity of the chromosomally encoded normal transhydrogenase.) Subsequently, we have mutated the remaining conserved polar residues of domain II. The catalytic and proton-translocating activities varied from 50% to near normal levels for all mutants, with the exception of those of βAsn^{222}. Catalytic activity and proton translocation were reduced to near background levels in βN222A. Although nothing is known about the organization of the transmembrane helices of domain II, it is worth noting that βHis^{91} and βAsn^{222} are at a similar level in the membrane, as modeled in Fig. 2. This suggests that helices 7 and 11 might interact to form the proton pathway.

Table 1
Catalytic and proton-pumping activities of membrane vesicles of mutants of the putative proton pathway

Mutant	Catalytic activity (%)	Proton-pumping activity (%)
Nonmutant	100	100
αH450T	17	51
αR502S	71	93
βE82Q	63	85
βE82K	79	66
βH91S	19	11
βH91T	11	8
βH91C	12	7
βH91K	4	20
βH91D	15	9
βH91N	80	7
βK145T	29	71
βH161S	90	100
βH161T	88	108
βH161C	59	94
βD213N	92	44
βD213H	82	34
βC260S	78	57

Catalytic and proton-pumping activities are expressed as percentages of the specific activity in nonmutant membranes. The data were taken from Refs. [26,32].

Mutation of some other semiconserved residues of domain II (Fig. 2) did not reveal further significant residues. Of interest, mutation of βGlu^{124}, the residue in the β subunit labelled by DCCD [27], reduced catalytic activity by 50% but had little effect on proton translocation.

In summary, of conserved charged and other polar residues, only βHis^{91} and βAsn^{222} are essential for normal catalytic and/or proton-translocation activities. The mutant βH91K contains tightly bound $NADP^+$ and the β subunit is susceptible to cleavage by trypsin at βArg^{265} without addition of NADP(H) [33]. As discussed in Section 1, proton pumping is probably driven by the difference in binding energies of NADPH and $NADP^+$. Thus, the properties of this mutant provide a linkage between the putative proton-translocating residue βHis^{91}, the binding of NADP(H) at the catalytic site, and the change in enzyme conformation on NADP(H) binding, which is probably associated with proton pumping. Also linking this residue with conformational change are the properties of the βH91C, T and S mutants. These mutants were unable to undergo the NADP(H)-induced conformational change [32]. Yamaguchi and Hatefi [34] have proposed that βAsp^{213} is involved in proton translocation since the βD213I mutant showed reduced catalytic and proton translocation activities, and had a higher affinity for NADPH. Several conserved acidic residues in domain III (βGlu^{361}, βGlu^{371}, βGlu^{374}, βAsp^{383}, βAsp^{392}) were mutated by Mueller et al. [35]. Only βAsp^{392} mutants were

significantly affected in catalytic activity and proton translocation.

5. Effect of deletions on assembly of the transhydrogenase

By studying a series of deletion mutants in which various regions of the transhydrogenase were deleted, it was shown that assembly of the β subunit into the enzyme was contingent on the insertion of α subunits into the membrane [36]. The highly nonpolar domain II region of the α subunit terminates in a 10-amino acid segment which is highly polar and contains four positively charged residues (Fig. 2). Premature termination of the α subunit six amino acid residues from the carboxy terminal end resulted in complete loss of enzyme activity. The subunits were assembled in the membrane, but the conformation of the β subunit was perturbed [37]. Systematic truncation and site-directed mutagenesis revealed that at least one positive charge is required in this region for efficient assembly of the two subunits to give a functional enzyme, and that αPhe^{507}, although not affecting assembly, was essential for activity. The role of the carboxy terminal region in the assembly of the β subunit was examined by introduction of chain-terminating codons into transhydrogenase-encoding plasmid DNA. Deletion of the two carboxy terminal amino acids (βAla^{461} and βLeu^{462}) prevented incorporation of both α and β subunits into the membrane. A series of site-directed mutants of these two amino acids was constructed. The mutations had relatively minor effects on catalytic activity. Thus, it is unlikely that these residues have a direct catalytic role but are important in maintaining the native conformation of the transhydrogenase.

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References

[1] M. Hutton, J.M. Day, T. Bizouarn, J.B. Jackson, Eur. J. Biochem. 219 (1994) 1041–1051.
[2] Y. Hatefi, M. Yamaguchi, FASEB J. 10 (1996) 444–452.
[3] O. Fjellström, C. Johansson, J. Rydström, Biochemistry 36 (1997) 11331–11341.
[4] D.M. Clarke, T.W. Loo, S. Gillam, P.D. Bragg, Eur. J. Biochem. 158 (1986) 647–653.
[5] S. Ahmad, N. Glavas, P.D. Bragg, Eur. J. Biochem. 207 (1992) 733–739.
[6] C. Hou, M. Potier, P.D. Bragg, Biochim. Biophys. Acta 1018 (1990) 61–66.
[7] T. Olausson, O. Fjellström, J. Meuller, J. Rydström, Biochim. Biophys. Acta 1231 (1995) 1–19.
[8] J.B. Jackson, N.P.J. Cotton, R. Williams, T. Bizouarn, M.N. Hutton, L.A. Sazanov, C.M. Thomas, Biochem. Soc. Trans. 21 (1993) 1010–1013.
[9] P.-S. Hu, B. Persson, J.-O. Höög, H. Jörnvall, A.F. Hartog, J.A. Berden, E. Holmberg, J. Rydström, Biochim. Biophys. Acta 1102 (1992) 19–29.
[10] M.G. Rossman, D. Moras, K.W. Olsen, Nature 250 (1974) 194–199.
[11] N.S. Scrutton, A. Berry, R.N. Perham, Nature 343 (1990) 38–43.
[12] O. Fjellström, T. Olausson, X. Hu, B. Källebring, S. Ahmad, P.D. Bragg, J. Rydström, Proteins Struct. Funct. Genet. 21 (1995) 91–104.
[13] P.D. Bragg, N.A. Glavas, C. Hou, Arch. Biochem. Biophys. 338 (1997) 57–66.
[14] P.D. Bragg, C. Hou, Eur. J. Biochem. 241 (1996) 611–618.
[15] S. Wakabayashi, Y. Hatefi, Biochem. Int. 15 (1987) 915–924.
[16] M. Yamaguchi, Y. Hatefi, K. Trach, J.A. Hoch, J. Biol. Chem. 263 (1988) 2761–2767.
[17] T. Olausson, T. Hultman, E. Holmberg, J. Rydström, S. Ahmad, N.A. Glavas, P.D. Bragg, Biochemistry 32 (1993) 13237–13244.
[18] N.A. Glavas, S. Ahmad, P.D. Bragg, T. Olausson, J. Rydström, J. Biol. Chem. 268 (1993) 14125–14130.
[19] N.A. Glavas, P.D. Bragg, Biochem. Mol. Biol. Int. 35 (1995) 297–306.
[20] A.P. Singh, P.D. Bragg, Can. J. Biochem. 57 (1979) 1384–1391.
[21] B. Persson, J. Rydström, Biochem. Biophys. Res. Commun. 142 (1987) 573–578.
[22] J. Meuller, J. Zhang, C. Hou, P.D. Bragg, J. Rydström, Biochem. J. 324 (1997) 681–687.
[23] E.G. Sedgwick, J. Meuller, C. Hou, J. Rydström, P.D. Bragg, Biochemistry 36 (1997) 15285–15293.
[24] J.D. Venning, R.L. Grimley, T. Bizouarn, N.P.J. Cotton, J.B. Jackson, J. Biol. Chem. 272 (1997) 27535–27538.
[25] R.C.W. Tong, N.A. Glavas, P.D. Bragg, Biochim. Biophys. Acta 1080 (1991) 19–28.
[26] E. Holmberg, T. Olausson, T. Hultman, J. Rydström, S. Ahmad, N.A. Glavas, P.D. Bragg, Biochemistry 33 (1994) 7691–7700.

[27] N.A. Glavas, C. Hou, P.D. Bragg, Biochem. Biophys. Res. Commun. 214 (1995) 230–238.
[28] H. Lüdi, W. Hasselbach, Eur. J. Biochem. 130 (1993) 5–8.
[29] K. Jung, H. Jung, J. Wu, G.G. Privé, H.R. Kaback, Biochemistry 32 (1993) 12273–12278.
[30] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Science 272 (1996) 1136–1144.
[31] E. Pebay-Peyroula, G. Rummel, J.P. Rosenbusch, E.H. Landau, Science 277 (1997) 1676–1681.
[32] N.A. Glavas, C. Hou, P.D. Bragg, Biochemistry 34 (1995) 7694–7702.
[33] N.A. Glavas, P.D. Bragg, Biochim. Biophys. Acta 1231 (1995) 297–303.
[34] M. Yamaguchi, Y. Hatefi, J. Biol. Chem. 270 (1995) 16653–16659.
[35] J. Meuller, X. Hu, C. Bunthof, T. Olausson, J. Rydström, Biochim. Biophys. Acta 1273 (1996) 191–194.
[36] S. Ahmad, N.A. Glavas, P.D. Bragg, J. Biol. Chem. 267 (1992) 7007–7012.
[37] S. Ahmad, N.A. Glavas, P.D. Bragg, J. Mol. Biol. 234 (1993) 8–13.