

agents that increase the intracellular cAMP concentration, leads to dephosphorylation of EF-2.

Another example of an *in vivo* system where reversible EF-2 phosphorylation is implicated is the mitogenic stimulation of cells. A dramatic transient increase of EF-2 phosphorylation has been observed<sup>5</sup> after mitogenic stimulation of fibroblasts, which ran parallel to but lagged slightly behind the intracellular Ca<sup>2+</sup> transient.

Parallel studies of the elongation rate changes were not done in any of the above examples. Nevertheless, these results, together with those reported here, strongly indicate that the reversible EF-2 phosphorylation may serve as a link between intracellular second messengers such as cAMP and the rate of translation.

Finally we would like to note that, after this paper was submitted, a communication by Nairn and Palfrey<sup>11</sup> reported that EF-2 from rat pancreas, that has been phosphorylated by EF-2 kinase (Ca<sup>2+</sup>/calmodulin-dependent protein kinase III) from the same source, is almost completely inactive in poly(U)-directed translation.

We are grateful to Professor A. S. Spirin for discussions and for critical reading of the manuscript. The help and advice of Drs E. K. Davydova, A. S. Sitikov and P. N. Simonenko in performing some experiments is also acknowledged.

Received 18 March; accepted 18 May 1988.

1. Palfrey, H. C. *FEBS Lett.* **157**, 183-190 (1983).
2. Nairn, A. C., Bhagat, B. & Palfrey, H. C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7939-7943 (1985).
3. Ryazanov, A. G. *FEBS Lett.* **214**, 331-334 (1987).
4. Ryazanov, A. G., Natapov, P. G., Shestakova, E. A., Severin, F. F. & Spirin, A. S. *Biochimie* (in the press).
5. Palfrey, H. C., Nairn, A. C., Muldoon, L. L. & Villereal, M. L. *J. biol. Chem.* **262**, 9785-9792 (1987).
6. Nairn, A. C., Nichols, R. A., Brady, M. J. & Palfrey, H. C. *J. biol. Chem.* **262**, 14265-14272 (1987).
7. Pain, V. M. *Biochem. J.* **235**, 625-637 (1986).
8. Sitikov, A. S., Simonenko, P. N., Shestakova, E. A., Ryazanov, A. G. & Ovchinnikov, L. P. *FEBS Lett.* **228**, 327-331 (1988).
9. Shestakova, E. A. & Ryazanov, A. G. *Dokl. Akad. Nauk SSSR* **297**, 1495-1498 (1987).
10. Hashimoto, S., Iwasaki, C., Kuzuya, H. & Guroff, G. *J. Neurochem.* **46**, 1599-1604 (1986).
11. Nairn, A. C. & Palfrey, H. C. *J. biol. Chem.* **262**, 17299-17303 (1987).
12. O'Farrell, P. H. *J. biol. Chem.* **250**, 4007-4021 (1975).

## Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes

T. de Vrije\*, R. L. de Swart†, W. Dowhan‡, J. Tommassen†§ & B. de Kruijff\*§

\* Department of Biochemistry, † Department of Molecular Cell Biology, § Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht, The Netherlands  
‡ Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas 77225, USA

Newly synthesized proteins to be exported out of the cytoplasm of bacterial cells have to pass across the inner membrane. In Gram-negative bacteria ATP<sup>1,2</sup>, a membrane potential<sup>3,4</sup>, the products of the *sec* genes<sup>5</sup> and leader peptidases<sup>6,7</sup> (enzymes which cleave the N-terminal signal peptides of the precursor proteins) are required. The mechanism of translocation, however, remains elusive. Important additional roles for membrane lipids have been repeatedly suggested both on theoretical grounds<sup>8-11</sup> and on the basis of experiments with model systems<sup>12-14</sup> but no direct evidence had been obtained. We demonstrate here, using mutants of *Escherichia coli* defective in the synthesis of the major anionic membrane phospholipids, that phosphatidylglycerol is involved in the translocation of newly synthesized outer-membrane proteins across the inner membrane.

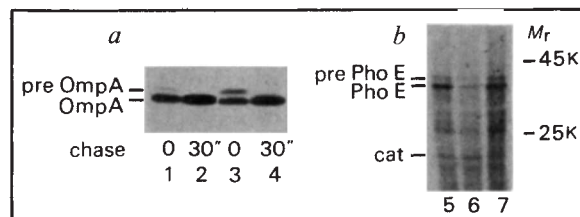
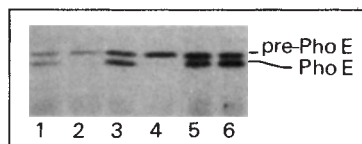


Fig. 1 Effect of PG and CL content on the *in vivo* synthesis and translocation of *E. coli* outer membrane proteins. *a*, Plasmid pHD102 containing cells of wild-type strain CE1224 (lanes 1 and 2, high PG and CL) and *pgsA3*-containing strain CE1250 (lanes 3 and 4, low PG and CL) were grown in a synthetic medium<sup>27</sup> at 43°C, until a low PG content was reached in CE1250. Cells were then pulse-labelled with [<sup>35</sup>S]methionine at 37°C for 30 s (ref. 28), followed by a chase with unlabelled methionine for the indicated time period. After trichloroacetic acid precipitation the samples were immunoprecipitated using rabbit antiserum against OmpA protein<sup>29</sup> and protein A coupled to Sepharose<sup>30</sup>. *b*, Strains SD12 (lane 5, wild-type), HD3122 (*pgsA3*) (lane 6, low PG and CL) and SD11 (lane 7, low CL), each containing plasmid pJP29 which carries the *phoE* gene<sup>28</sup>, were induced for PhoE protein synthesis by growth at 37°C in low phosphate medium<sup>31</sup>, supplemented with chloramphenicol (25 µg ml<sup>-1</sup>). Whole cells were pulse-labelled with [<sup>35</sup>S]methionine for 10 s at 30°C. Strain CE1250 was derived from *E. coli* K12 strain CE1224<sup>32</sup>, containing pHD102, by P1 transduction<sup>33</sup> using strain HD3122 which carries a *Tn10* insertion close to the *pgsA3* allele as donor. A tetracycline-resistant transformant with a low PG content after growth at 43°C was designated CE1250. Curing plasmid pHD102 from this strain followed by plating resulted in pinpoint colonies, indicating that loss of an intact *pgsA* allele is harmful in this genetic background. All samples were analysed by SDS-polyacrylamide gel electrophoresis<sup>34</sup> and the dried gels were subjected to fluorography. The position of some relative molecular mass (*M<sub>r</sub>*) standard proteins are shown on the right. For determination of the phospholipid composition, phospholipids were isolated from whole cells or membrane vesicles by solvent extraction<sup>35</sup> and separated by thin-layer chromatography on silica gel using chloroform:methanol:acetic acid (65:25:10) as solvent one and chloroform:pyridine:formic acid (60:30:7) as solvent two. The phosphate content in each spot was measured after perchloric acid destruction<sup>36</sup>.

Phosphatidylglycerol phosphate synthase is a key enzyme in the biosynthesis of phosphatidylglycerol (PG), the major acidic phospholipid in *E. coli*. A mutation in the *pgsA* locus of the *E. coli* genome was described<sup>15</sup>, which resulted in undetectable levels of activity of this enzyme and consequently very low levels of PG and cardiolipin (CL), which is derived from PG. Several *E. coli* strains carrying this *pgsA3* allele required the presence of a plasmid (such as pHD102) carrying a functional *pgsA* gene for growth<sup>16</sup>, suggesting that these lipids are very important. We used such *pgsA3 E. coli* strains to study the role of the anionic lipids in protein translocation.

Cells of the *pgsA3 E. coli* strain CE1250 were cured of plasmid pHD102 by growth at the restrictive temperature for plasmid replication (43°C). When the PG content dropped to 1-3 mol% of the total phospholipids (analysed as described in Fig. 1), the cells were pulse-labelled with [<sup>35</sup>S]methionine to follow the biosynthesis of the outer membrane protein OmpA. Immunoprecipitation showed that significantly more precursor (pre-OmpA) was present, compared to the wild-type strain with normal PG levels (Fig. 1*a*, lanes 1 and 3). In both cases the precursor disappeared during a 30-second chase (Fig. 1*a*, lanes 2 and 4). This is the first indication that the rate of translocation of an outer membrane protein across the inner membrane is decreased by reduced levels of anionic phospholipids.

The *E. coli* strain SD12 carrying the *pgsA3* allele is able to grow normally<sup>16</sup> (strain HD3122) although in agreement with previous reports, the mutation caused a reduction in PG and CL from 11 to 1.0 and 3-4 to 0.5 mol% of total phospholipid respectively. This drop in total level of anionic phospholipids



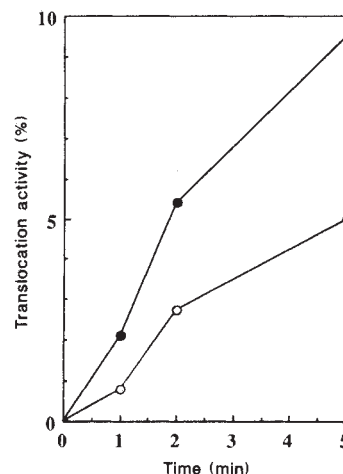
**Fig. 2** Effects of low PG or CL content in inner-membrane vesicles on the processing and translocation of *in vitro* synthesized pre-PhoE. Transcription *in vitro* of plasmid pJP29 which carries the *phoE* gene and synthesis of [<sup>35</sup>S]methionine labelled pre-PhoE protein were as described previously<sup>24</sup>. Translation was carried out for 27.5 min at 37° C using a S-135 cell extract of strain SD12 (lanes 1 and 2) or HD3122 (lanes 3–6). SD12 (lanes 1, 3, 5), HD3122 (lanes 2, 4) or SD11 (lane 6) vesicles were added 7.5 min after the translation had been started to a final concentration of 0.5  $A_{280}U ml^{-1}$ , corresponding to 0.2  $\mu mol$  phospholipid phosphorus per ml. Protease digestion was carried out by adding 200  $\mu g ml^{-1}$  of proteinase K for 30 min at 22° C and translocated proteins were analysed by fluorography of 12% SDS-polyacrylamide gels. Radioactivity of the protein bands was determined<sup>24</sup>. Inner-membrane vesicles were prepared<sup>24</sup> from whole cells grown in yeast broth at 37° C (lanes 1–4; ref. 27) or 30° C (lanes 5 and 6). In SD11 cells grown at 30° C the CL content is  $\sim 0.05 mol\%$  of the total phospholipids.

is slightly counterbalanced by an accumulation of phosphatidic acid (1.8%). No significant change in lipid/protein ratio occurred in the inner membrane (0.4  $\mu mol$  lipid per mg protein), and nor did the fatty acid composition of the total cellular phospholipids change drastically (7% increase in 18:1c at the expense of 16:1c).

The plasmid pJP29, carrying the *phoE* gene, was introduced into strains SD12 and HD3122 and these were then induced for the synthesis of the major outer membrane pore protein PhoE. Pulse-labelling of the cells showed that more precursor (pre-PhoE) accumulated relative to processed product (PhoE) in the mutant strain HD3122, than in the wild-type strain (Fig. 1b, lanes 5 and 6). Total synthesis of PhoE protein, but not of the cytoplasmic protein chloramphenicol acetyl transferase (CAT), was reduced during the pulse in HD3122. In addition, cells containing the *pgsA3* allele had an elongated shape. Such decreased synthesis of secreted proteins and an elongated shape are often observed in export-defective *E. coli* cells<sup>17–21</sup>. To discriminate between effects of PG and CL on protein translocation similar experiments were conducted with strain SD11, which is defective in CL synthase<sup>22</sup>. These cells, when grown at 37° C, have a normal PG but a very low CL content (0.1 mol% of total phospholipid). Compared to the wild-type strain SD12, total PhoE synthesis and translocation were similar (Fig. 1b, lane 7), strongly suggesting that it is the reduced level of PG that is responsible for the effects observed with the *pgsA3*-allele carrying strains.

Cell-free translation-translocation systems<sup>23</sup> allow a more detailed analysis of the influence of low acidic-phospholipid levels on protein translocation. Translocation of PhoE protein across inverted *E. coli* inner-membrane vesicles can be monitored by processing of translocated pre-PhoE by endogenous leader peptidase and by its protection against added protease<sup>24</sup>.

Pre-PhoE protein was synthesized in such a system supplemented with a cytosolic extract of the wild-type SD12 strain and vesicles of either SD12 or HD3122 (low PG, low CL). Translocation across mutant vesicles was reduced as shown by a decrease in the percentage of total synthesized PhoE which was insensitive to protease digestion because it was sequestered in the vesicles (7.5% and 3.4% for SD12 and HD3122 vesicles, respectively), and by an inhibition of pre-PhoE processing as indicated by the low intensity of the PhoE band (Fig. 2, lanes 1 and 2; processing of translocated pre-PhoE was 56% and 21% for SD12 and HD3122 vesicles, respectively). These effects are not due to the low CL content as there was efficient translocat-



**Fig. 3** Kinetics of post-translational translocation of PhoE protein across inner-membrane vesicles of strain SD12 (●) and HD3122 (○). Synthesis of pre-PhoE was carried out for 25 min at 37° C using a cell extract of strain HD3122, after which translation was inhibited by the antibiotic puromycin<sup>24</sup>. To 40  $\mu l$  of the translation system, 10  $\mu l$  containing ATP (20 mM), unlabelled methionine (500  $\mu M$ ) and an optimal amount of inner-membrane vesicles for translocation was added (final concentration 0.5  $A_{280}U ml^{-1}$ ) and the incubation was continued at 37° C. At the indicated points of time translocation was stopped by the addition of proteinase K and the translocation activity was determined.

tion and processing of PhoE in SD11 vesicles (Fig. 2, lanes 5 and 6; percentage translocated protein was 30% and 24% for SD11 and control vesicles, respectively). Because the effect of the low PG level on PhoE translocation and processing appears to be stronger *in vitro* than *in vivo* (compare Figs 1 and 2), we considered whether a cytoplasmic factor in the HD3122 lysate might compensate for the decrease in PG content. This appears to be the case, as the mutant lysate produced a threefold stimulation of translocation activity of both SD12 and HD3122 vesicles (Fig. 2, lanes 3 and 4; 20.5% and 8.8% total translocation for SD12 and HD3122 vesicles, respectively). The nature of this factor is not known.

Figure 3 confirms that the mutant HD3122 vesicles are less effective in pre-PhoE translocation than the wild-type strain in a post-translational translocation experiment. Moreover, these data directly show that it is the rate of translocation which is decreased by lowering the PG level. After 5 min translocation levels off, and it even decreases after longer incubation times, presumably due to digestion of the proteins. The efficiency of processing of the translocated pre-PhoE was independent of time. Both SD12 and HD3122 vesicles generated a membrane potential (measured as described in ref. 24) which was sufficient for maximal translocation activity and dissipation of which by 5  $\mu M$  CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone) reduced translocation to a similar low level. Therefore, we assume that the involvement of PG in protein translocation is not the indirect result of changes in membrane potential.

Although protein processing and translocation need not be strictly coupled processes in *E. coli*<sup>25</sup>, it could be argued that inhibition of processing causes blocking of export sites, thereby reducing the translocation efficiency of pre-PhoE. Consistent with this suggestion, the activity of purified *E. coli* leader peptidase on pre-PhoE, in a mixed micellar  $\beta$ -octyl glucoside detergent system, was stimulated two to fivefold by PG but not by the zwitterionic phosphatidylethanolamine or phosphatidylcholine. Translocation itself, and not just processing, however, is affected in the HD3122 vesicles as deduced from experiments with a PhoE mutant protein, deleted in the N-terminal mature part from amino-acid position +3 to +18, which is translocated

*in vivo* across the inner membrane into the periplasmic space without being processed<sup>26</sup>. This mutant protein is also translocated across wild-type and HD3122 vesicles without being processed. Translocation across mutant vesicles was reduced 40% compared to wild-type vesicles, demonstrating that lack of processing alone is not responsible for inhibition of translocation (data not shown).

Whether PG acts in the protein translocation pathway across the *E. coli* inner membrane in a direct or indirect manner is not yet known. Recent model membrane-experiments<sup>14</sup> with the signal peptide of PhoE and *E. coli* membrane lipids revealed a negatively-charged lipid-specific insertion of the signal peptide into the membrane, resulting in changes in both signal peptide structure and lipid organization. This is in agreement with a role for signal-peptide/lipid interactions for protein translocation across the *E. coli* inner membrane.

We thank Dr Shibuya and D. Bosch for gifts of strain SD11 and the plasmid carrying the deleted PhoE gene, respectively. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO), and by the United States Public Health Service awarded to W.D.

Received 6 May; accepted 27 May 1988.

- Chen, L. & Tai, P. C. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4384-4388 (1985).
- Chen, L. & Tai, P. C. *Nature* **328**, 164-166 (1987).
- Date, T., Goodman, J. M. & Wickner, W. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4669-4673 (1980).

- Bakker, E. P. & Randall, L. L. *EMBO J.* **3**, 895-900 (1984).
- Benson, S. A., Hall, M. N. & Silhavy, T. J. *Rev. Biochem.* **54**, 101-134 (1985).
- Wolfe, P. B., Wickner, W. & Goodman, J. M. *J. Biol. Chem.* **258**, 12073-12080 (1983).
- Hussain, M., Ichihara, S. & Mizushima, S. *J. Biol. Chem.* **257**, 5177-5182 (1982).
- Wickner, W. *Science* **210**, 861-868 (1980).
- Pollitt, S., Inouye, S. & Inouye, M. *Microbiology* **1986** 238-241 (1986).
- Nesmeyanova, M. A. *FEBS Lett.* **142**, 189-193 (1982).
- De Kruijff, B. *et al.* in *The Enzymes of Biological Membranes* (ed. Martonosi, A.) 131-204 (Plenum, New York, 1985).
- Ohno-Iwashita, Y., Wolfe, P., Ito, K. & Wickner, W. *Biochemistry* **23**, 6178-6184 (1984).
- Briggs, M. S., Cornell, D. G., Dluhy, R. A. & Gierasch, L. M. *Science* **233**, 206-208 (1986).
- Batenburg, A. M., Demel, R. A., Verkleij, A. J. & De Kruijff, B. *Biochemistry* (in the press).
- Miyazaki, C., Kuroda, M., Ohta, A. & Shibuya, I. *Proc. natn. Acad. Sci. U.S.A.* **82**, 7530-7534 (1985).
- Heacock, P. N. & Dowhan, W. *J. Biol. Chem.* **262**, 13044-13049 (1987).
- Hall, M. N., Gabay, J. & Schwartz, M. *EMBO J.* **2**, 15-19 (1983).
- Strauch, K. L., Kumamoto, C. A. & Beckwith, J. *J. Bact.* **165**, 181-192 (1986).
- Baker, K., Mackman, N., Jackson, M., & Holland, I. B. *J. molec. Biol.* **198**, 693-703 (1987).
- Oliver, D. B. & Beckwith, J. *Cell* **25**, 765-772 (1981).
- Hall, M. N., Schwartz, M. & Silhavy, T. J. *J. molec. Biol.* **156**, 93-112 (1982).
- Shibuya, I., Miyazaki, C. & Ohta, A. *J. Bact.* **161**, 1086-1092 (1985).
- Müller, M. & Biobel, G. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7737-7741 (1984).
- De Vrije, T., Tommassen, J. & De Kruijff, B. *Biochim. biophys. Acta* **900**, 63-72 (1987).
- Dalbey, R. E. & Wickner, W. *J. Biol. Chem.* **260**, 15925-15931 (1985).
- Bosch, D., Voorhout, W. & Tommassen, J. *J. Biol. Chem.* (in the press).
- Lugtenberg, B., Peters, R., Bernheimer, H. & Berendsen, W. *Molec. gen. Genet.* **147**, 251-262 (1976).
- Bosch, D. *et al.* *J. molec. Biol.* **189**, 449-455 (1986).
- Overbeeke, N., Van Scharrenburg, G. & Lugtenberg, B. *Eur. J. Biochem.* **110**, 247-254 (1980).
- Ito, K., Bassford, P. J. & Beckwith, J. *Cell* **24**, 707-717 (1981).
- Tommassen, J., Leunissen, J., Van Damme-Jongsten, M. & Overduin, P. *EMBO J.* **4**, 1041-1047 (1985).
- Tommassen, J., Van Tol, H. & Lugtenberg, B. *EMBO J.* **2**, 1275-1279 (1983).
- Willets, N. S., Clark, A. J. & Low, K. B. *J. Bact.* **97**, 244-249 (1969).
- Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. & Van Alphen, L. *FEBS Lett.* **58**, 254-258 (1975).
- Bligh, E. G. & Dyer, W. J. *Can. J. Biochem. Physiol.* **37**, 911-917 (1959).
- Rouser, G., Fleischer, S. & Yamamoto, A. *Lipids* **5**, 494-496 (1984).

## X-ray structure of a DNA hairpin molecule

Rajagopal Chattopadhyaya\*, Satoshi Ikuta\*, Kazimierz Grzeskowiak & Richard E. Dickerson†

Department of Chemistry & Biochemistry and Molecular Biology Institute, University of California, Los Angeles, California 90024, USA

We have solved the crystal structure of a synthetic DNA hexadecanucleotide of sequence: C-G-C-G-C-G-T-T-T-C-G-C-G-C-G, at 2.1 Å resolution, and observed that it adopts a monomeric hairpin configuration with a Z-DNA hexamer stem. In the T<sub>4</sub> loop the bases stack with one another and with neighbouring molecules of the crystal, and not with base pairs of their own hexamer stem. Two thymine T10 rings from different molecules stack between the C1-G16 ends of a third and a fourth hairpin helix, in a manner that suggests T-T base 'pairing' and simulates a long, 13-base-pair helix. Although such T-T interactions would not be present in solution, they illustrate a remarkable tendency of thymines for self-association. Purine-purine G-A base pairs are known to exist in the *anti-anti* conformation with an increase in local helix width<sup>1-4</sup>; it may be that more serious consideration should be given to the possible existence of pyrimidine-pyrimidine C-T base pairs with decreased local helix width, particularly where several such base pairs occur sequentially.

The 16-mer structure was solved by molecular replacement, using idealized B-DNA and Z-DNA hexamers to search for the position of the hairpin stem in the crystal. The Z helix gave better agreement with the X-ray data than the B, and was selected for further refinement. As Figs 1 and 2 show, the six-base-pair stem is a normal left-handed Z-DNA helix, like those observed in the crystal structures of C-G-C-G-C-G (ref. 5) or <sup>13</sup>C-G-<sup>13</sup>C-G-<sup>13</sup>C-G (ref. 6). Glycosyl bond conformations alternate between

*anti* at Cs and *syn* at Gs. Deoxyribose ring conformations are in the C2'-*endo* family at Cs and the C3'-*endo* family at Gs, except for the 3'-terminal guanine sugars at the two ends of the Z stem. These are C2'-*endo*, as is generally found at this position in Z structures. The adoption by interior guanines of C3'-*endo* sugar puckering appears to be a requirement for a continuing Z-helical backbone chain, rather than any intrinsic preference for *syn* guanines for the C3'-*endo* conformation. Even at G6 in the hairpin hexadecamer, where the chain continues but in the form of a loop rather than a Z helix, the guanine sugar conformation relaxes from C3'-*endo* to C2'-*endo*.

The growth of crystals of DNA hairpins has been intractable in the past. This is probably because monomer hairpins are an intermediate state in a three-state equilibrium bounded on one side by isolated random-coil chains, and on the other by double helices<sup>7-10</sup>. The shift from hairpin monomers to helical duplexes is favoured by lower temperature and by higher DNA and salt concentrations, all of which also are conditions favouring crystal growth. The present C-G-C-G-C-G-T-T-T-C-G-C-G-C-G may be particularly favoured for hairpin crystallization because its sequence permits it to adopt another high-concentration, high-salt alternative to the duplex: a Z helix.

The four-base-pair loop is close to the minimum sterically acceptable length<sup>11-13</sup>. The path of the chain was determined by difference-map methods, and given a final check by the difference map shown in Fig. 3a. There is little sign of any tendency for loop bases to stack against the final G-C base pair of the helix. The only element of the loop that stacks against base pair G6-C11 is S9, the sugar ring of thymine 9. In place of such stacking on G6-C11, the loop builds a ministack of its own consisting of sugar S7 and thymine rings T8 and T9 at right angles to the base planes of the stem (Fig. 3b).

Sugar O4' atoms have a tendency to nest against neighbouring aromatic rings. It is a characteristic feature of Z-DNA that each cytosine sugar-oxygen is packed against the guanine ring that follows it in a 5'-3' direction. In the present structure, G6 is sandwiched between the oxygens of sugar S5 in the stem and S8 in the loop. The oxygen of S7 nests against T8, and that of S9 against S8. Only with S10 is the deoxyribose sugar exposed to the surroundings.

\* Present addresses: Department of Biochemistry, University of California, Berkeley, California 94720, USA (R.C.); Department of Chemistry, Illinois Institute of Technology, Chicago, Illinois 60616, USA (S.I.).

† To whom correspondence should be addressed.