Determinants of Helix-Loop-Helix Dimerization Affinity

RANDOM MUTATIONAL ANALYSIS OF SCL/tal

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Dimerization represents a key regulatory step in the function of basic helix-loop-helix (bHLH) transcriptional factors. In many instances tissue-specific basic helix-loop-helix proteins, such as the hematopoietic factor SCL/tal or the myogenic factor MyoD, interact with ubiquitously expressed basic helix-loop-helix proteins, such as E2A or E2-2. Such dimerization is necessary for high affinity, sequence-specific DNA binding. Previous biochemical and structural studies have shown the helix-loop-helix region to be necessary and sufficient for this interaction. In the present study, we analyzed the relative affinities of various helix-loop-helix interactions using the yeast two-hybrid system. The relative affinities of selected helix-loop-helix species for the partner protein E2-2 were as follows: Id2 > MyoD > SCL/tal. Mutants of SCL/tal with increased affinity for E2-2 were selected from a library of randomly mutated basic helix-loop-helix domains. The amino acid changes in these high affinity versions of SCL/tal introduced residues that resembled those in the corresponding positions of the Id proteins and MyoD. One of the mutants, SCL 12, also contained mutations in highly conserved residues previously thought to be necessary for dimerization. This mutant of SCL demonstrated diminished temperature sensitivity in in vitro interaction assays as compared with the wild type protein. Computational modeling of helix-loop-helix dimers provides an explanation for the increased dimerization affinity of SCL mutant 12.

Basic helix-loop-helix (bHLH) transcriptional factors play a fundamental role in cell fate determination in eukaryotic organisms ranging from Caenorhabditis elegans to humans. This family of over 60 different proteins has been implicated in processes such as lineage commitment, differentiation programming, cell cycle regulation, and oncogenesis (1–5). Many of these activities arise from sequence-specific DNA binding by bHLH factors, followed by transcriptional activation of target genes. The structural and biochemical features of HLH dimerization have not been thoroughly characterized. X-ray crystallographic studies indicate that the HLH regions dimerize in the form of a parallel four-bundle left-handed helix, with many of the dimerization contacts occurring within a hydrophobic core region (6–8). Biological studies indicate that for some dimers, e.g., E2A homodimers, intermolecular disulfide bond formation may be important in stabilizing interactions (18). Biochemical studies of MyoD heterodimerization with E12, using site-directed mutagenesis, indicate a role for non-conserved charged residues in helix 2 of MyoD forming ionic bonds with charged residues in helix 1 of E12 (19). Similar studies have identified a homodimerization inhibitory domain, amino-terminal to the basic domain in E12, which directs preferential heterodimerization with MyoD (20).

To further characterize the determinants of helix-loop-helix dimerization, we have employed the yeast two-hybrid system to analyze the heterodimerization of the hematopoietic bHLH protein SCL/tal with the E protein E2-2. E2-2 was chosen as the E protein partner because of its preferential formation of heterodimers with SCL as compared with E2-2 homodimer formation (11). In addition the E2-2 bHLH domain is highly homologous (95%) to that of E47, for which a crystal structure...
is available (6). The yeast two-hybrid system permits direct and accurate quantitation of protein-protein interactions (21) and has previously been applied toward analyzing helix-loop-helix dimerization (22). In our studies Id and MyoD proteins, as compared with SCL/tal, displayed significantly higher affinity for E2-2. The bHLH domain of SCL/tal was subjected to random mutagenesis, and mutants with normal or increased affinity for E2-2 were selected. Several of the changes in the high affinity SCL/tal mutants introduced amino acids identical or similar to those in the corresponding positions of the Id and MyoD proteins. One of the mutants, SCL/tal 12, contained non-conservative amino acid changes in the two most highly conserved positions in the bHLH family. Using an in vitro interaction assay, we found that the increased affinity of SCL 12 for E2-2 displayed a temperature dependence, manifesting at 30°C but not at 4°C. Computational modeling of SCL/tal binding to E proteins provides an explanation for the properties of SCL/tal mutant 12.

MATERIALS AND METHODS

Plasmid Constructions—The SCL/tal cDNA was provided by Ilan Kirsch (NCI, Bethesda, MD). The MyoD cDNA was provided by Harold Weintraub (Center for Cancer Research, Bethesda, MD). The E2-2 cDNA was provided by Tom Kadesch (University of Pennsylvania School of Medicine, Philadelphia, PA). The yeast two-hybrid analysis were kindly provided by the laboratory of Roger Brent (Massachusetts General Hospital, Boston, MA) (23). The yeast two-hybrid system permits direct and indirect binding to E proteins providing an explanation for the properties of SCL/tal mutant 12.

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The E2-2 cDNA was provided by Tom Kadesch (University of Pennsylvania School of Medicine, Philadelphia, PA). The yeast two-hybrid system permits direct and indirect binding to E proteins providing an explanation for the properties of SCL/tal mutant 12.

RESULTS

Relative Affinities of HLH Proteins for E2-2—To compare affinities of various HLH domains for the common partner protein E2-2, the yeast two-hybrid system was employed. In this system, the "bait" consists of the E2-2 bHLH domain produced as a fusion protein with the LexA DNA binding domain, and the "prey" consists of prey plasmids transformed into yeast containing the LexA DNA binding domain (21). The yeast two-hybrid system permits direct and indirect binding to E proteins providing an explanation for the properties of SCL/tal mutant 12.
HLH preys, consisting of fusions of MyoD, Id2, and SCL/tal with the B42 transcriptional activation peptide, were coexpressed via yeast mating with the LexA-E2-2 bait protein. The preys were expressed as fusions of the B42 transcriptional activator domain with the respective HLH domains (wild type SCL/tal, Id2, and MyoD). The bait consisted of the LexA DNA binding domain fused to the bHLH domain of the E2-2 protein. Prey and bait plasmids, transformed into separate yeast strains (YPH-499 and EGY48-pSH18-34, respectively), were brought together through mating. Within the resultant diploid strains activation of the lacZ reporter plasmid, pSH18-34, was quantitated with liquid β-galactosidase assays, which were repeated on three different occasions. Western blotting was employed to verify prey expression in yeast. Equivalent quantities of yeast from A were subjected to SDS-PAGE followed by Western blot with the 12CA5 monoclonal antibody. 12CA5 recognizes an epitope (hemagglutinin) present in all of the prey proteins.

HLH preys, consisting of fusions of MyoD, Id2, and SCL/tal with the B42 transcriptional activation peptide, were coexpressed via yeast mating with the LexA-E2-2 bait protein. Quantitative β-galactosidase assays were performed on equivalent numbers of yeast for each interaction. The relative affinities for E2-2 are shown in Fig. 1A. Using SCL/tal as a standard of comparison, MyoD has a slightly increased affinity for E2-2 (8.5-fold), and Id2 has considerably increased affinity for E2-2 (65.2-fold). These results correlate with previously published data using the Far Western blotting system, in which Id1 had greater affinity than SCL/tal for E2-2 (11). To further confirm that the results of the β-galactosidase assays reflected the affinities of the various preys for the E2-2 bait, as opposed to differential expression levels of the various preys, prey expression in the various strains was assayed by Western blotting. Using a monoclonal antibody (12CA5) that recognizes an epitope tag common to all preys, Western blot shows roughly equivalent levels of expression of all the preys (Fig. 1B).

Screening a Library of Randomly Mutated SCL/tal for Variants with Enhanced Affinity for E2-2—To determine which residues might play a role in the enhanced affinity of Id2 and MyoD for E2-2, SCL/tal was subjected to random mutagenesis using error-prone PCR (24). SCL/tal mutants with enhanced affinity for E2-2 were selected by using the yeast two-hybrid mating/screening approach (23). Table I shows the relevant parameters in this library screening. When wild type SCL/tal was employed as the prey, 2% of diploid yeast (which contain prey, bait, and reporters) manifested growth on leucine-deficient medium. When randomly mutated SCL/tal was employed as prey, only 0.07% of diploid yeast manifested growth on leucine-deficient medium. Therefore, an estimated 97% of the SCL/tal clones obtained by random mutagenesis have sustained mutations eliminating the capacity for dimerization with E2-2. Among those clones that retained the capacity for dimerization with E2-2, the majority displayed affinities similar to that of wild type SCL/tal. One such clone, SCL/tal mutant 4, was selected for further analysis. Three of the clones from the SCL/tal mutant pool (clones 9, 12, and 36) showed significantly enhanced affinity for E2-2 and were also selected for further study.

The relative affinities for E2-2 and the amino acid sequences of the SCL/tal mutants are displayed in Fig. 2A. SCL/tal mutant 4, with similar affinity for E2-2 as wild type SCL/tal, contained a single amino acid change, K234E in helix 2. SCL/tal mutant 12 contained a total of four amino acid changes, two in helix 1 (N204D and G205E) and two in helix 2 (K225E and K234E). Two of the amino acid changes in SCL/tal mutant 12 are conserved residues in the HLH family, which normally interact to form an intramolecular hydrogen bond (6, 13). SCL/tal mutant 9, with moderately increased affinity for E2-2, contains a total of two amino acid changes, one in helix 1 (N202D) and one in helix 2 (M233I). An alignment of the high affinity SCL/tal mutants with MyoD and the Id family is shown in Fig. 2B. The residues shared in common by the high affinity SCL/tal mutants and the MyoD and Id proteins are highlighted. From this alignment, it is evident that the majority of amino acid changes in the high affinity SCL/tal mutants introduce residues that are similar or identical to residues at corresponding positions in MyoD and the Id proteins.

Synergy of Mutations in Helix 1 and Helix 2 of High Affinity SCL/tal Mutants—In an attempt to determine which specific amino acid changes were responsible for the increased affinity for E2-2, chimeras between wild type and mutants of SCL/tal were analyzed for E2-2 binding. The sequences and E2-2 binding affinities of the various chimeras are shown in Fig. 3A. For SCL/tal mutant 9 neither the mutation in helix 1 (N202D) nor the mutation in helix 2 (M233I) alone can fully account for the increased affinity for E2-2 seen in the intact mutant. In fact, the relationship between the mutations in helix 1 and helix 2 of SCL/tal mutant 9 appears to be synergistic rather than additive. Similarly, for SCL/tal mutant 12 the mutations in helix 1 show a synergistic interaction with the mutations in helix 2 in augmenting the affinity for E2-2. Equivalent expression of SCL/tal wild type, mutants, and chimeras was documented by Western blot analysis (Fig. 3B). From these data we conclude that while single amino acid changes may modestly enhance

**TABLE I**

| Prey         | Relative lacZ Units |
|--------------|---------------------|
| SCL/tal wt   | 1                   |
| Id2          | 65.2±1.5            |
| MyoD         | 8.5±3.4             |

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**Fig. 1.** A, relative quantitation of HLH interactions using the yeast two-hybrid technique. The preys were expressed as fusions of the B42 transcriptional activator domain with the respective HLH domains (wild type SCL/tal, Id2, and MyoD). The bait consisted of the LexA DNA binding domain fused to the bHLH domain of the E2-2 protein. Prey and bait plasmids, transformed into separate yeast strains (YPH-499 and EGY48-pSH18-34, respectively), were brought together through mating. Within the resultant diploid strains activation of the lacZ reporter plasmid, pSH18-34, was quantitated with liquid β-galactosidase assays, which were repeated on three different occasions. B, Western blot analysis of prey expression in yeast. Equivalent quantities of yeast from A were subjected to SDS-PAGE followed by Western blot with the 12CA5 monoclonal antibody. 12CA5 recognizes an epitope (hemagglutinin) present in all of the prey proteins.

**TABLE I**

| Conditions                        | Yield of diploids with wt SCL/tal prey showing leucine prototrophy: |
|----------------------------------|------------------------------------------------------------------|
| No. of library yeast transformants: | 80,000                                                           |
| No. of diploids with bait strain:  | 16,000                                                           |
| No. of leucine prototrophic colonies after 4 days: | 11                                                              |
| No. of leucine prototrophic colonies after 4 days: | 0.1%                                                             |

**TABLE I**

| Conditions                        | Yield of diploids with mut SCL/tal prey showing leucine prototrophy: |
|----------------------------------|------------------------------------------------------------------|
| No. of library yeast transformants: | 80,000                                                           |
| No. of diploids with bait strain:  | 16,000                                                           |
| No. of leucine prototrophic colonies after 4 days: | 11                                                              |
| No. of leucine prototrophic colonies after 4 days: | 0.07%                                                            |

**TABLE I**

| Conditions                        | Yield of diploids with mut SCL/tal prey showing leucine prototrophy: |
|----------------------------------|------------------------------------------------------------------|
| No. of library yeast transformants: | 80,000                                                           |
| No. of diploids with bait strain:  | 16,000                                                           |
| No. of leucine prototrophic colonies after 4 days: | 11                                                              |
| No. of leucine prototrophic colonies after 4 days: | 2.0%                                                             |
the affinity of SCL/tal for E2-2, marked enhancement of this affinity requires amino acid changes in both helix 1 and helix 2.

The Enhanced E2-2 Binding of Mutant 12 Is Only Manifested at Higher Temperatures—To verify the enhanced binding to E2-2 by SCL/tal mutant 12, in vitro protein interaction assays were performed with 32P-labeled soluble E2-2 and immobilized GST fusion proteins. Fig. 4A (lanes 1 and 2) illustrates binding of soluble [32P]E2-2 to the carboxyl-terminal half of SCL/tal (amino acids 200–331) and absence of binding of [32P]E2-2 to SCL/tal lacking an HLH domain (amino acids 255–331). At 4°C, the bHLH domain of wild type SCL/tal (lane 3) binds E2-2 with slightly increased affinity as compared with the bHLH domain of SCL/tal mutant 12 (lane 4). By contrast, when the interaction assay is carried out at 30°C (Fig. 4B), SCL/tal mutant 12 (lanes 2, 4, and 6) shows significantly enhanced binding to [32P]E2-2 as compared with wild type SCL/tal (lanes 1, 3, and 5). As shown in Fig. 4B, the relative enhancement of [32P]E2-2 binding by SCL/tal mutant 12 at 30 °C occurs across a range of concentrations of E2-2. These results indicate a greater thermal stability for E2-2 dimerization with SCL/tal mutant 12 as opposed to E2-2 dimerization with SCL/tal wild type.

DISCUSSION

The Id HLH proteins are potent dominant negative inhibitors of bHLH proteins, exerting their effects through the se-

FIG. 2. A, amino acid sequences of the HLH domain of wild type and mutant SCL/tal proteins. The SCL/tal mutants were selected, using the yeast two-hybrid approach, from a pool of randomly mutated HLH domains. Binding of these SCL/tal mutants to E2-2 is quantified by measuring the activation of the lacZ reporter plasmid as described in Fig. 1. The affinities are expressed relative to those of seven independent yeast colonies containing wild type SCL/tal prey. B, alignment of HLH domains from MyoD, Id proteins, wild type SCL/tal, and mutants of SCL/tal. Relative affinities for E2-2, as measured by the yeast two-hybrid system, are displayed as relative lacZ units. Relative lacZ units for each interaction were calculated from three independent β-galactosidase assays. The numbering of amino acid residues derives from the SCL/tal HLH domain. Amino acid residues shown in bold are those shared by the high affinity SCL/tal mutants and MyoD and Id proteins.

FIG. 3. A, analysis of chimeras between wild type and mutant SCL/tal HLH domains. The indicated SCL/tal HLH domains were assayed for E2-2 binding as described in Figs. 1 and 2. B, Western blot analysis of prey protein expression. Equivalent quantities of yeast from Fig. 3A were subjected to SDS-PAGE followed by Western blot with the 12CA5 monoclonal antibody as described in Fig. 1B.
determinants in HLH interactions. The amino acid alignment affinities, comparable to those of the Id and MyoD proteins, were analyzed by SDS-PAGE/autoradiography. A, binding of [32P]E2-2 to GST fusion proteins at 4°C. In lane 1, the GST fusion contains the intact bHLH domain of SCL/tal as well as the carboxyl terminus, amino acids 200–331. In lane 2, the bHLH domain has been deleted from SCL/tal. In lane 3, the GST fusion contains the minimal bHLH domain of wild type SCL/tal, amino acids 186–242. In lane 4, the GST fusion contains the minimal bHLH domain of SCL/tal mutant 12. B, binding of [32P]E2-2 to GST fusion proteins at 30°C. In lanes 1, 3, and 5, the GST fusion contains the minimal bHLH domain of wild type SCL/tal. In lanes 2, 4, and 6, the GST fusion contains the minimal bHLH domain of SCL/tal mutant 12. Varying quantities of [32P]E2-2, as indicated in the figure, were combined with the immobilized GST fusion proteins.

One of the amino acid changes in the high affinity SCL/tal mutants, G205E, introduces an acidic residue into helix 1 at a position where many other HLH proteins contain an acidic residue: MyoD and Iδ2 (Fig. 2B), as well as E12, E47, E2-2, HEB, Daughterless, Twist, myogenin, and MRF4. The crystallographic model of dimerization in Fig. 5A suggests that this amino acid is not close enough to E2-2 to form a bond (8.3 Å from Arg-199 of E2-2). However, previous biochemical studies clearly indicate that an acidic residue at this specific position strongly contributes to dimerization, possibly by forming an electrostatic bond with a basic residue in helix 2 of the partner protein (19). A similar effect might arise from the N202D mutation in helix 1 of SCL/tal mutant 9: Id1, Id3, E12, E47, E2-2, HEB, Daughterless, and Myc all possess an acidic residue at this position.

The M233I mutation in helix 2 of SCL/tal mutant 9 increases the hydrobicity at this position. Methionine has a Kyte-Doolittle index (Kd) value of 1.9, and isoleucine has a Kd value of 4.5. Notably, most HLH proteins, including the Id, myogenic, E protein, and achaete-scute families, possess a highly hydrophobic residue at this position, either isoleucine (Kd value of 4.5) or valine (Kd value of 4.2). Structural data from x-ray crystallography indicate that the residue at this position contributes to a hydrophobic core at the dimerization interface (6). Correspondingly, we have shown that by simply changing methionine 233 to isoleucine one can reproducibly increase the affinity of SCL/tal for E2-2 by 3-fold.

In the SCL/tal mutant (mutant 12) with highest affinity for E2-2, asparagine 204 and lysine 225 are replaced by acidic residues, aspartic acid and glutamic acid, respectively. These changes represent non-conservative substitutions at the two most highly conserved residues in the entire HLH family (13). To analyze the structural consequences of these mutations, computational modeling was performed using the crystallographic coordinates of the E47 homodimer (see “Materials and Methods”). The model shows that N204 and K225 normally interact to form an intramolecular hydrogen bond, approximating helix 1 and helix 2 of SCL/tal (Fig. 5A). Our experimental data indicate that despite stringent evolutionary conservation, these residues are not required for HLH dimerization. Fig. 5 (B and C) shows a model predicting the effects of the combined N204D and K225E mutations in SCL/tal. By introducing acidic amino acids at positions 204 and 225 in SCL/tal, the intramolecular hydrogen bond between helix 1 and helix 2 is disrupted. However, the glutamic acid residue at position 225 of SCL/tal is optimally oriented to form an electrostatic bond with the guanido group of arginine 199 in helix 1 of the partner E protein. The net result is that an intramolecular hydrogen bond is lost and an intermolecular ionic bond is gained. A functional corollary is that monomeric SCL/tal, destabilized by the loss of an intramolecular bond, becomes much less energetically favorable than the heterodimeric form which is stabilized by introduction of an additional intermolecular ionic bond. From this model, one might predict that heterodimers of E2-2 with SCL/tal mutant 12 would show increased thermal stability, as compared with heterodimers of E2-2 with wild type SCL/tal (see data in Fig. 4). In order to confirm our hypothetical model, it will be necessary to perform direct crystallographic analyses on complexes of E2-2 with wild type and mutant versions of SCL/tal.

Our results show that the affinity of SCL/tal for E2-2 has not been maximized by natural evolution. Excessive affinity for an E protein partner may represent an undesirable property from an evolutionary standpoint. This excessive affinity might alter the functional characteristics, e.g. DNA binding, of SCL/tal-E protein complexes. Alternatively, the excessive affinity may diminish the reversibility of HLH complex formation, essen-
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In many systems of cellular differentiation, rapid dynamic alterations in the arrays of HLH complexes are required at various developmental stages (3, 12). Usage of high affinity dimerization mutants of HLH proteins in these systems may provide insight into the function of normally transient, rapidly reversible HLH complexes.

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REFERENCES

1. Olson, E. N., and Klein, W. H. (1994) Genes & Dev. 8, 1–8
2. Weintraub, H. (1994) Cell 75, 1241–1244
3. Jän, Y. N., and Jän, L. Y. (1993) Cell 75, 827–830
4. Bain, G., Robanus Maandag, E. C., Izon, D. J., Amsen, D., Kruisbeek, A. M., Weintraub, B. C., Krap, H., Schnabel, S. M., Feeley, A. J., van Roon, M., van der Valk, M., te Riele, H. P. J., Bens, A., and Murrie, C. (1994) Cell 79, 885–892
5. Brown, L., Cheng, J.-T., Chen, Q., Siciliano, M. J., Crist, W., Buchanan, G., and Baer, R. (1990) EMBO J. 9, 3343–3351
6. Ellenberger, T., Fass, D., Arnaud, M., and Harrison, S. C. (1994) Genes & Dev. 8, 970–980
7. Ma, P. C. M., Roul, M. A., Weintraub, H., and Pabo, C. O. (1994) Cell 77, 451–459
8. Davis, R. L., Cheng, P.-F., Lassar, A. B., and Weintraub, H. (1990) Cell 60, 733–746
9. Murre, C., McCaw, P. S., Vaessen, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Haushka, S. D., Lassar, A. B., Weintraub, H., and Baltimore, D. (1989) Cell 58, 537–544
10. Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Versonov, A., Baltimore, D., and Weintraub, H. (1991) Cell 66, 305–315
11. Godtberg, A. N., and Lewandowska, K. (1995) Blood 85, 465–471
12. Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y., and Lassar, A. (1995) Science 263, 761–766
13. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990) Cell 61, 49–59
14. Sun, X.-H., Copeland, N. G., Jenkis, N. A., and Baltimore, D. (1991) Mol. Cell. Biol. 11, 5603–5611
15. Sun, X.-H. (1994) Cell 79, 893–900
16. Jen, Y., Weintraub, H., and Benezra, R. (1992) Genes & Dev. 6, 1466–1479
17. Shoji, W., Yamamoto, T., and Ohnata, M. (1994) J. Biol. Chem. 269, 5078–5084
18. Benezra, R. (1994) Cell 79, 1057–1067
19. Shirakata, M., Friedman, F. K., and Paterson, B. M. (1993) Genes & Dev. 7, 2456–2470
20. Shirakata, M., and Paterson, B. M. (1995) EMBO J. 14, 1766–1772
21. Yang, M., Wu, Z., and Fields, S. (1995) Nucletic Acids Res. 23, 1125–1126
22. Staudinger, J., Perry, M., Elledge, S. J., and Olson, E. N. (1993) J. Biol. Chem. 268, 4608–4611
23. Zervos, A. S., Gyriris, J., and Trent, R. (1993) Cell 72, 223–232
24. Leung, D. W., Chen, E., and Goodell, D. V. (1989) Technique 1, 11–15
25. Gietz, D., Saint-Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Nucleic Acids Res. 20, 1425
26. Brent Laboratory (1993) Yeast Two-hybrid Procedures Manual. Available Gopher: Massachusetts General Hospital Molecular Biology Internet Gopher Server
27. Jones, T. A. (1978) J. Appl. Crystallogr. 11, 269–272
28. Jones, T. A., Jen, Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. 47, 110–119
29. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
30. Merrill, E. A., and Murphy, M. E. (1994) Acta Crystallogr. 50, 869–873