“Cold-Sensitive” Mutants of the Lac Repressor[▽]

Andrew Barker,^{1,2} Stefan Oehler,^{1,3*} and Benno Müller-Hill¹

Institut für Genetik der Universität zu Köln, Zùlpicherstr. 47, D-50674 Köln, Germany¹; Western Australian Institute for Medical Research, Rear 50 Murray Street, Perth, WA 6000, Australia²; and IMBB-FORTH, Vassilika Vouton, P.O. Box 1378, GR-71110 Heraklion, Crete, Greece³

Received 15 September 2006/Accepted 6 December 2006

Thirteen of more than 4,000 single-amino-acid-replacement mutants of the Lac repressor, generated by suppression of amber nonsense mutants, were characterized as having a cold-sensitive phenotype. However, when expressed as missense mutations, none of the replacements cause cold sensitivity, implicating the suppression mechanism as being responsible for this phenotype.

The Lac repressor (LacR) is one of the best analyzed proteins. Its three-dimensional structure is known at high resolution (4, 10). More than 4,000 single-amino-acid-replacement mutants have been analyzed (6, 11). Among these, 13 cold-sensitive mutants were found (Fig. 1). The single most striking feature of these mutants is their distribution: 11 out of 13 amino acid replacements in six out of eight codons (of a total data set of 4,042 single substitutions covering residues 2 to 329 of the 360-amino-acid long LacR) are located in the DNA-binding headpiece (11). These amino acid replacements have been generated by *in vivo* suppression of amber nonsense mutants using suppressor tRNAs of the indicated specificity (11). This clustering seems to suggest that in these cold-sensitive mutants, regions in the DNA binding domain of the Lac repressor “freeze” at low temperatures into stable structures which are unable to bind properly to the *lac* operator (1).

Interested in the biochemical properties of these mutants, we introduced appropriate missense mutations into plasmid pWB1000, which constitutively produces about 400 times the *lacI*⁺ amount of wild-type (tetrameric) LacR (8, 9). In order to verify the cold-sensitive nature of the introduced mutations, the ability of each mutant protein to repress a chromosomal *lacIO*⁺*p*⁺*Z*⁺*Y*⁺ allele in *trans* was tested at 37°C and 25°C. Repression values and induction with the gratuitous inducer isopropyl-β-D-thiogalactopyranoside (IPTG) were determined by β-galactosidase assays of the relevant strains (6, 8, 11, 12, 13). We were surprised to see only borderline cold-sensitive phenotypes (Table 1), with a maximum twofold-greater repression at 37°C than at 25°C for S16C, V20C, V23A, H29A, H29E, K33H, K33F, and K33E. Some alleles even show heat sensitivity, with a 12-fold-greater repression for R35E or a threefold-greater repression for E259F at 25°C.

Since overexpression of LacR might compensate for cold sensitivity, a series of strains was constructed, in which each of the cold-sensitive LacR variants is produced at the wild-type level (about 10 tetramers per cell). The reporter system used consists of a chromosomal copy of *lacZ* under control of a *lacI* gene integrated at the attachment site for bacteriophage λ

(15). Repression values were determined by comparing *lacZ* expression in the presence or absence of the gratuitous inducer IPTG at 37°C or 25°C to expression in the absence of any LacR. Here, only one mutation, S16A, shows a borderline cold sensitivity, with about 1.6-fold-weaker repression at 25°C than at 37°C. R35E retains a clear heat-sensitive phenotype, with a roughly eightfold-reduced repression at 37°C (in addition to being substantially reduced in its ability to repress even at 25°C), while V23C and H29E (both with 1.9-fold-reduced repression) and K33F (with 1.6-fold-reduced repression) show weak heat sensitivity. All mutants are fully inducible at both 25°C and 37°C, insofar as it is possible to measure induction of the essentially null mutant E259F (data not shown). Thus, not only are none of the 13 alleles cold sensitive, but neither V150C nor E259F has a *LacI*^s phenotype, contrary to earlier reports (11, 14, 16).

We also tested a subset of the mutations for cold sensitivity *in vitro*. Partially purified extracts containing LacR alleles S16A, V20C, H29A, and K33E were prepared, and their ability to bind *lacO*_{id}-containing plasmid DNA was compared to that

FIG. 1. Cold-sensitive missense suppression variants of LacR presented by Miller and coworkers (11, 16). The protein sequence of the N terminus of LacR plus the two residues of the core protein for which there are cold-sensitive amino acid replacements are shown. Positions where replacements (indicated above the sequence) can lead to a cold-sensitive phenotype are in boldface. The residues of the DNA binding recognition helix (residues 17 to 26) are in italic. The indicated replacements were generated by suppression of an amber mutation at the respective position of an episomally encoded *lacI*^s allele, using a suppressor tRNA of the indicated specificity (6, 11). The *lacZ* reporter was also episomally encoded in these strains.

* Corresponding author. Mailing address: IMBB-FORTH, Vassilika Vouton, P.O. Box 1378, GR-71110 Heraklion, Crete, Greece. Phone: 30 2810-391144. Fax: 30 2810-391104. E-mail: oehler@imbb.forth.gr.

[▽] Published ahead of print on 15 December 2006.

TABLE 1. In vivo activities of missense LacR mutants at 25°C and 37°C^a

LacR	Repression value ^b			
	400× <i>lacI</i> ⁺ ^c		1× <i>lacI</i> ⁺ ^d	
	25°C	37°C	25°C	37°C
Wild type	16,000	17,000	1,900	1,900
S16C	8,800	17,000	6.7	5.9
S16A	1,300	1,700	19	31
V20C	1,600	3,000	100	74
V23C	25,000	34,000	1,900	1,000
V23A	22,000	39,000	1,300	1,000
H29A	16,000	34,000	2,000	1,500
H29E	1,200	1,900	230	120
K33H	25,000	54,000	1,100	840
K33F	12,000	27,000	520	320
K33E	2,900	6,800	190	190
R35E	530	44	12	1.5
V150C	40,000	54,000	2,300	1,900
E259F	4,700	1,400	1.8	2.4

^a Detailed experimental procedures and background information are available on request.

^b The repression value is defined as the quotient of the specific activity of β -galactosidase in the absence of any active repressor and the specific activity of β -galactosidase in the presence of the indicated LacR variant. A repression value of 1 indicates no repression.

^c The indicated LacR variants were expressed from derivatives of pWB1000 transformed into strain JMX8634R (relevant genotype, *lacIO*⁺*p*⁺*Z*⁺*Y*⁺) to a level of about 4,000 LacR tetramers per cell. Values obtained in the absence of any active LacR were obtained in the presence of pWB100Δ1, which encodes a LacR derivative in which codons 14 to 60 are deleted and which is thus defective for DNA binding (8).

^d The indicated LacR variants were expressed from the appropriate *lacI* allele inserted at *attλ* of strain JMX8634R. Although these constructs differ from wild-type *lac* in that *lacI* is not adjacent to *lacOpZY* but is instead nearly 10 min (i.e., roughly 450 kb) away (2), levels of LacR are the same as in the wild-type situation, that is, about 10 tetramers per cell (15). Values obtained in the absence of any active LacR were obtained from the background strain JMX8634R without any *lacI* allele integrated at *attλ*. All alleles were fully inducible with IPTG.

of wild-type LacR in equilibrium binding studies (1, 3). No differences were detected in filter-binding experiments at 4°C, 25°C, or 37°C (data not shown).

These unexpected results prompted us to verify the results obtained by Miller and coworkers (11, 16). Strains bearing the relevant *lacI*(Am) nonsense mutations and suppressors were obtained from Miller, and their identities were confirmed by DNA sequencing. We were largely able to reproduce the results of Miller and coworkers (data not shown).

It thus appears that there are no cold-sensitive mutants of LacR. Given the wealth of characterized amino acid replacements for all positions of the headpiece and core protein, it seems justified to assume that no single amino acid replacement can turn the Lac repressor into a cold-sensitive protein.

There are, on the other hand, well-documented cases of true cold-sensitive mutants of other proteins (5, 7). The case of the Lac repressor should be helpful in determining which features of a protein make it amenable to cold sensitivity.

One question remains to be answered: how does the apparent cold sensitivity of the suppressed nonsense mutants arise? Wild-type Lac repressor has restarts at codons GUG23, GUG38, AUG42, and UUG62. Several of these have been removed in some alleles used by Miller et al., but all alleles retain the restart at position 42 (6). Suppression of nonsense codons leads to the expression of a certain fraction of full-

length protein, while some to most ribosomes still stop but can reinitiate translation at a downstream start codon. Restart products of Lac repressor core protein which lack the DNA binding domain will form inactive mixed oligomers with the full-length repressor and thus be dominant negative when expressed in sufficient amounts. One could argue that their concentration increases at low temperature. However, this is obviously not the full explanation. Why are there cold-sensitive nonsense mutations at positions 16 and 20 but not at positions 17, 18, 19, and so on? The cold-sensitive mutants of the Lac repressor remain, on a different level, an enigma.

We are grateful to Jeffrey Miller and to Tony Poteete for their generous provision of materials used and to Jeffrey Miller for commenting on an earlier version of the manuscript. We thank Karin Schnetz for providing laboratory space.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemie.

REFERENCES

- Barker, A., and B. Müller-Hill. 1998. Towards a mutant analysis of the tertiary structures of functional DNA-binding motifs. *FEBS Lett.* **432**:1–3.
- Berlyn, M., K. B. Low, and K. E. Rudd. 1996. Linkage map of *Escherichia coli* K-12, edition 9, p. 1715–1902. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. H. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, DC.
- Dong, F., S. Spott, O. Zimmermann, B. Kisters-Woike, B. Müller-Hill, and A. Barker. 1999. Dimerisation mutants of Lac repressor I. A monomeric mutant, L251A, that binds *lac* operator DNA as a dimer. *J. Mol. Biol.* **290**:653–666.
- Kalodimos, C. G., A. M. J. J. Bonvin, R. K. Salinas, R. Wechselberger, R. Boelens, and R. Kaptein. 2002. Plasticity in protein-DNA recognition: *lac* repressor interacts with its natural operator *O*₁ through alternative conformations of its DNA-binding domain. *EMBO J.* **21**:2866–2876.
- Kermekchiev, M. B., A. Tzekow, and W. B. Barnes. 2003. Cold-sensitive mutants of Taq DNA polymerase provide a hot start for PCR. *Nucleic Acids Res.* **31**:6139–6147.
- Kleina, L. G., and J. H. Miller. 1990. Genetic studies of the *lac* repressor. XIII. Extensive amino acid replacements generated by the use of natural and synthetic nonsense suppressors. *J. Mol. Biol.* **212**:295–318.
- Laursen, B. S., I. Siwanowicz, G. Larigauderie, J. Hedegaard, K. Ito, Y. Nakamura, J. M. Kenney, K. K. Mortensen, and H. U. Sperling-Petersen. 2003. Characterisation of mutations in the GTP-binding domain of IF2 resulting in cold-sensitive growth of *Escherichia coli*. *J. Mol. Biol.* **326**:543–551.
- Lehming, N., J. Sartorius, M. Niemöller, G. Genenger, B. von Wilcken-Bergmann, and B. Müller-Hill. 1987. The interaction of the recognition helix of *lac* repressor with *lac* operator. *EMBO J.* **6**:3145–3153.
- Lehming, N., J. Sartorius, S. Oehler, B. von Wilcken-Bergmann, and B. Müller-Hill. 1988. Recognition helices of *lac* and λ repressor are oriented in opposite directions and recognise similar DNA sequences. *Proc. Natl. Acad. Sci. USA* **85**:7947–7951.
- Lewis, M., G. Chang, N. C. Horton, M. A. Kercher, H. C. Pace, M. A. Schumacher, R. G. Brennan, and P. Lu. 1996. Crystal structure of the lactose operon repressor and its complexes with DNA and inducer. *Science* **271**:1247–1254.
- Markiewicz, P., L. G. Kleina, C. Cruz, S. Ehret, and J. H. Miller. 1994. Genetic studies of the *lac* repressor. XIV. Analysis of 4000 altered *Escherichia coli* *lac* repressors reveals essential and non-essential residues, as well as “spacers” which do not require a specific sequence. *J. Mol. Biol.* **240**:421–433.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Oehler, S., E. R. Eismann, H. Krämer, and B. Müller-Hill. 1990. The three operators of the *lac* operon cooperate in repression. *EMBO J.* **9**:973–979.
- Pace, H. C., M. A. Kercher, P. Lu, P. Markiewicz, J. H. Miller, G. Chang, and M. Lewis. 1997. Lac repressor genetic map in real space. *Trends Biochem. Sci.* **22**:334–339.
- Spott, S., F. Dong, B. Kisters-Woike, and B. Müller-Hill. 2000. Dimerisation mutants of Lac repressor II. A single amino acid substitution, D278L, changes the specificity of dimerisation. *J. Mol. Biol.* **296**:673–684.
- Suckow, J., P. Markiewicz, L. G. Kleina, J. Miller, B. Kisters-Woike, and B. Müller-Hill. 1996. Genetic studies of the Lac repressor. XV. 4000 single amino acid substitutions and analysis of the resulting phenotypes on the basis of the protein structure. *J. Mol. Biol.* **261**:509–523.