Protein Modifications in the D2 Protein of Photosystem II Affect Properties of the Q_B/Herbicide-Binding Environment

Hadar Kless[a], Michal Oren-Shamir[b], Itzhak Ohad[c], Marvin Edelman[b], and Wim Vermaas[a]

[a] Department of Botany and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, Arizona 85287-1601, U.S.A.
[b] Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel
[c] Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Z. Naturforsch. **48c,** 185–190 (1993); received November 23, 1992

Herbicides, Plastoquinone, Photosystem II, Thermoluminescence, Cyanobacteria

The D2 protein contains an extended loop (the *D-de* loop) between helices *D* and *de* at the reducing side of photosystem II (PS II). Characterization of D2 mutants of the cyanobacterium *Synechocystis* sp. PCC 6803 has indicated that the length and amino acid composition of the *D-de* loop are not critical for basic PS II functions, although most of the residues in that region are conserved phylogenetically. Here we show using herbicide binding and electron-flow inhibition measurements that drastic modifications in the *D-de* loop of the D2 protein modify the interaction of some PS II-directed herbicides with their binding niche. The stability of (semi-)reduced Q_B in its binding pocket is altered in at least two of the mutants, as indicated by a shifted peak temperature of the thermoluminescence signal originating from charge recombination involving Q_B.

These results suggest a close functional association between the *D-de* loop of the D2 protein and the Q_B/herbicide-binding environment, which is viewed as being coordinated mostly by residues of the D1 protein. This represents one of the first examples of modification of the Q_B/herbicide-binding domain by mutations in the D2 protein.

Introduction

Protein loop sequences in surface regions of proteins often are involved in binding and recognition [1]. In photosystem II (PS II), extended loop regions occur at several locations in its two reaction center protein components, D1 and D2. These include the *D-de* loop, which connects the transmembrane helix *D* and parallel helix *de* in each of these proteins [2, 3]. The *D-de* loop of D2 was speculated to be located near the binding pocket of the primary quinone (Q_A), and was suggested to be in close contact with the *D-de* loop of D1 [4], which is located in the vicinity of the Q_B/herbicide-binding niche. These *D-de* loop regions are highly conserved in both proteins, with 72% identity in all PS II-containing species characterized thus far [5].

The cyanobacterium *Synechocystis* sp. PCC 6803 was used recently to engineer the protein sequence of the *D-de* loop of the D2 protein [6]. A *psb*D frameshift mutant was isolated, and upon selection and analysis of photoautotrophic suppressor mutants it was revealed that dramatic changes in composition, length and net charge of the *D-de* loop in the D2 protein interfere only mildly with basic PS II functions [6]. Here we present a further characterization of these mutants by means of herbicide binding, herbicide inhibition of DCPIP reduction, and thermoluminescence. The results indicate that changes in the *D-de* loop region of D2 affect the Q_B/herbicide-binding environment, which is assumed to be created mostly by residues of the D1 protein.

Reprint requests to Dr. H. Kless.

Verlag der Zeitschrift für Naturforschung, D-W-7400 Tübingen
0939–5075/93/0300–0185 $ 01.30/0

Materials and Methods

Strains and growth conditions

Synechocystis sp. PCC 6803 cells were grown in BG-11 medium in the presence of 5 mM glucose as described [6, 7]. The wild type strain for these studies lacks *psb*D II, which was replaced by a spectinomycin resistance cartridge, and contains a kanamycin resistance cartridge downstream of *psb*D I/C [6, 8].

Transformation and nucleic acid manipulations

Transformation of *Synechocystis* sp. PCC 6803 [9] and site-directed mutagenesis [10, 11] were per-

formed as described. Generation of the photoautotrophic intragenic suppressor mutant lines D2S-A, -C, -E, -K, and -N is detailed in [6].

The sequence of these photoautotrophic mutants has been presented in Fig. 1. D2R is essentially comparable to wild type, except that a codon for arginine was inserted between nucleotides 669 and 670 of *psb*D1 (numbering according to ref. [12]). This insertion led to the creation of a unique *Nru*I site, which was used in further constructions to introduce the *D-de* region from D1 into this area of D2 [6].

Herbicide-binding assay

Quantitation of PS II on a chlorophyll (Chl) basis, and determination of the diuron affinity in whole cells was performed by [^{14}C]diuron binding as described [16].

Herbicide inhibition assay

Oxygen-evolving membranes were prepared as described [13, 14]. The rate of electron transfer from H_2O to the electron acceptor 2,6-dichlorophenolindophenol (DCPIP) was measured in thylakoids in T-buffer (50 mM HEPES pH 7.0; 5 mM $MgCl_2$; 50 mM $CaCl_2$; 5% (v/v) glycerol and 0.5% (v/v) DMSO) at a chlorophyll concentration of 5 μg/ml. Measurements were performed at room temperature and in the presence of 0.3 mM DCPIP [15]. DCPIP reduction was followed as the 590 nm absorption decrease over time upon illumination with saturating actinic light; this light was filtered through a RG 645 (Schott) glass filter before reaching the sample.

Thermoluminescence measurements

Cells were harvested in the late log phase, washed once in 25 mM HEPES (pH 7.0), and resuspended in the same buffer but containing 15% glycerol. Samples of 15 μg Chl/ml were used for thermoluminescence measurements. After dark adaptation for 2 min at 20 °C, a certain number of flashes was given at −5 °C, and the sample was frozen quickly to −40 °C. Light emission detected upon heating the cells at a rate of 0.7 °C/s was then measured. Where indicated, diuron was added to a final concentration of 50 μM to follow charge recombination between Q_A and the S_2/S_3 states.

Results

The sequence of the relevant portion of the D2 protein of the various mutants used in this study is presented in Fig. 1. The mutant D2R was constructed to have an additional codon of arginine to make the D2 sequence similar to that of D1 in that region. The D2S mutant series has originated by selection for natural photoautotrophic suppressor mutants of a photosynthetically incompetent mutant having a one-nucleotide deletion in the *D-de*-coding sequence of the *psb*D1 gene [6]. Various deletions and insertions have restored the reading frame resulting in drastic modification of the protein sequence in that loop region (Fig. 1).

D2R has the same growth rate as wild type cells, and its oxygen evolution rate is 88 ± 30% as compared to wild type (not shown). The photosynthetic capabilities of the suppressor (D2S) lines are reduced only mildly as compared to wild type

Fig. 1. Amino acid sequences of part of the D2 protein of wild type (residues 209–255), D2R, and the intragenic suppressor lines. This part of the protein corresponds to the region between the *D* and *de* helices (overlined). The modified residues in the *D-de* loop region are indicated by bold letters. A duplicated piece of 31 residues (corresponding to 94 nucleotides from an upstream region of the gene) is inserted at the indicated location (between R and G, underlined) in D2S-E. The sequences are aligned to match with the residues in both helices.

[6]. Photoautotrophic growth of these mutants ranged between 80 and 100%, and saturated rates of oxygen evolution ranged between 56 and 71% of the wild type rates.

PS II quantitation and diuron affinity

The dissociation constant of diuron and the number of herbicide-binding sites on a chlorophyll basis were determined by a [^{14}C]diuron-binding assay (Fig. 2 and Table I). These revealed a similar amount of PS II in D2R and wild type cells, and a reduced amount (47–80%) in the D2S lines. The dissociation constant of diuron in the mutants was found to be up to 2-fold larger than that in wild type, indicating changes in the intrinsic diuron affinity in these mutants.

Effect of herbicides on electron transfer

The effect of various herbicides on the rate of electron transfer from water to DCPIP was monitored in thylakoids of wild type and some of the mutants. The molar concentration of herbicide required for 50% inhibition of DCPIP reduction (I_{50} values) of the mutants was compared to that of wild type (Table II). The mutants have an up to threefold increase in the resistance towards diuron, while they generally are more resistant to atrazine (up to 10-fold more resistant as compared to wild type). Electron transfer is more sensitive to BNT (by 3–10-fold) in all of the mutants.

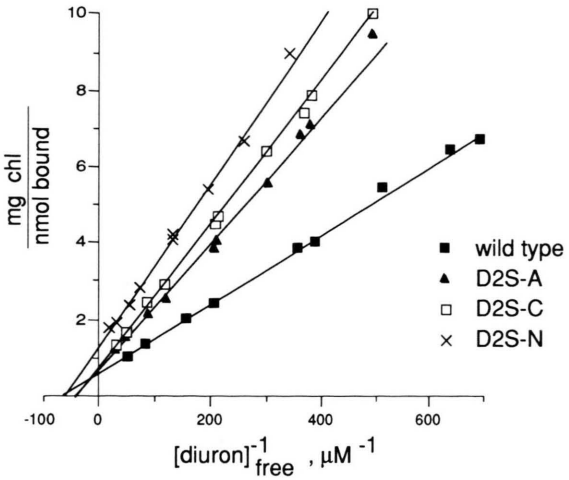

Fig. 2. Representative example of double-reciprocal plot of [^{14}C]diuron binding to cells of wild type and several of D2S mutants. The intersect with the Y-axis is proportional to the number of chlorophylls per PS II complex. The intersect with the X-axis reflects the negative inverse of the diuron dissociation constant *in vivo*.

Thermoluminescence

A convenient way to measure changes in the stability and redox midpoint potential of Q_B^- is by temperature-dependent chlorophyll luminescence upon heating a sample that is frozen in different redox states of the PS II complex. This process, thermoluminescence (TL), has been covered in some detail by DeVault and Govindjee [17] and Inoue [18]. In wild type *Synechocystis*, TL peaks

Table I. Diuron binding in wild type *Synechocystis* sp. PCC 6803 and D2 mutant cells.

Cell line[a]	Chlorophyll/ binding sites[b]	%[c]	K_d [nM][d]
WT	620 ± 155 (n = 6)	100	16 ± 3
D2R	600 ± 50 (n = 2)	103	30 ± 3.5
D2S-C	1038 ± 162 (n = 3)	60	29 ± 4.5
D2S-A	916 ± 290 (n = 3)	68	34 ± 14
D2S-K	780 ± 97 (n = 4)	80	15 ± 1.5
D2S-N	1334 ± 392 (n = 5)	47	23 ± 8
D2S-E	1310 ± 210 (n = 4)	47	16 ± 2.2

[a] See Fig. 1 for description of mutant lines.
[b] The number of herbicide-binding sites on a chlorophyll basis evaluate the amount of PS II in the thylakoids of intact cells. Average values ± standard errors for the indicated number of measurements are given in mg chlorophyll/nmol of bound herbicide.
[c] The precentage of diuron-binding sites relative to the wild type (100%) assuming one main-binding site per PS II.
[d] The dissociation constant of diuron reflects the inverse affinity of the herbicide to its binding pocket.

Table II. Herbicide inhibition of DCPIP reduction in thylakoid membranes of wild type and D2 mutants[a].

Cell line[b]	Diuron	Atrazine	BNT
WT[c]	1	1	1
D2R	2	2	0.2
D2S-C	3	5	0.1
D2S-A	3	10	0.2
D2S-E	3	9	0.3

[a] Resistances are given as relative I_{50} values (mutant/wild type).
[b] Designation of mutant cell lines are described in Fig. 1.
[c] Molar concentrations of I_{50} values of wild type are: 3×10^{-8}, 3.3×10^{-7}, and 1.2×10^{-7} for diuron, atrazine, and BNT, respectively.

occur at 10 °C for charge recombination when Q_A is reduced, while TL is emitted at 30–33 °C upon charge recombination involving Q_B^-. Under our conditions, in *Synechocystis* wild type as well as in most mutants, the integrated TL intensity observed after one flash is much smaller than that seen after two flashes (S. Carpenter, I. Ohad, and W. Vermaas, submitted). This could be due to a high Q_B^-/Q_B ratio after dark adaptation, or to the presence of one-electron acceptors or donors that are highly stable in reduced and oxidized conditions, respectively. Also, the peak temperature of TL after one flash is a few degrees higher than that after two flashes (reviewed in [17, 18]), presumably reflecting the different thermodynamic stability of the S_2 and S_3 states of the water-splitting system. Fig. 3A shows that wild type indeed shows a rather typical TL behavior. However, in all three measured D2S mutants the TL pattern has been modified. The maximum of the TL curve of mutant D2S-C has been shifted to 8–9 °C higher temperatures both after one and two flashes (Fig. 3A). This indicates a significant stabilization of reduced Q_B in this mutant. It is important to note that under our experimental conditions the TL amplitude of D2S-C after one flash was smaller relative to the second flash than was observed in wild type. As shown in Fig. 3B, the TL pattern of the D2S-E mutant is similar to that seen in D2S-C, but with an even more pronounced decrease in relative TL intensity after one flash, and increase in TL temperature: in D2S-E, after one flash very little TL is observed (Fig. 3B), even if diuron is present (data not shown). In this mutant, the TL peak after the second flash is at approximately 42 °C, which is about 12 °C higher than in wild type. This suggests that the mutation in D2 causes a significant increase in the stability of the Q_B semiquinone, implying an effect of the *D-de* loop of the D2 protein on the Q_B-binding environment. The glow peaks of D2S-A associated with recombination involving Q_B are similar to that seen in wild type; however, the 10–15 °C peak reflecting Q_A^- recombination is very pronounced in this mutant, even in the absence of herbicide (Fig. 3B).

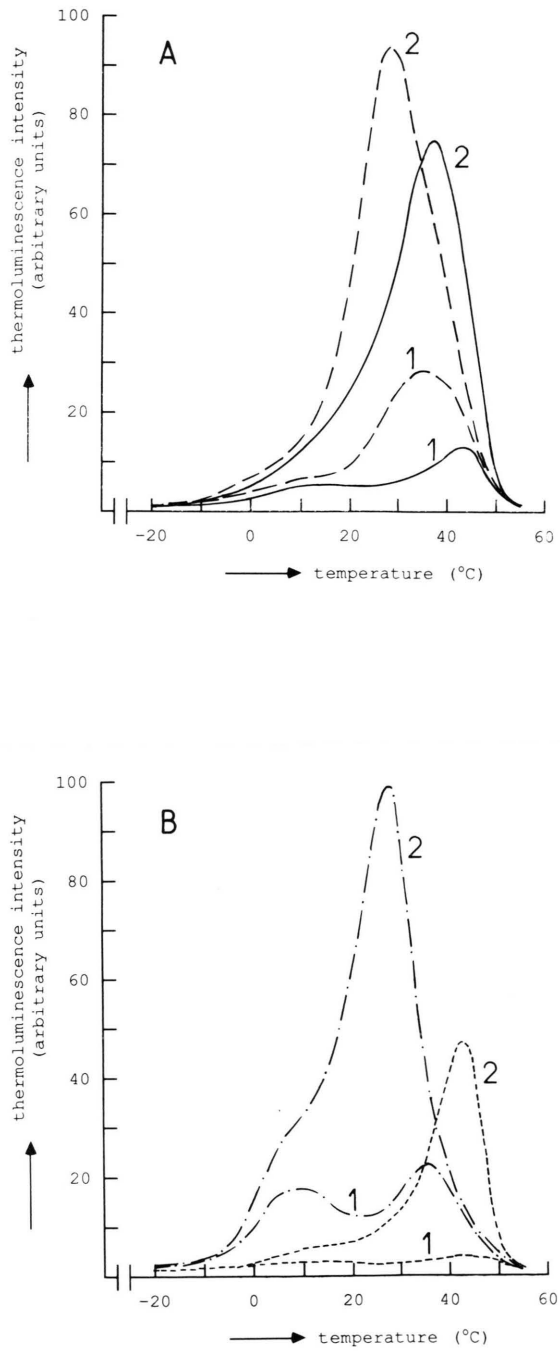

Fig. 3. Thermoluminescence glow curves measured in intact cells after one (1) or two (2) flashes, as indicated. Strains used: in Fig. A, – –: wild type; ——: D2S-C; in Fig. B, –·–·: D2S-A; ----: D2S-E. Typical thermoluminescence intensities detected (measured at the maximum after two flashes) were 11,500 cps for wild type, 10,000 cps for D2S-A, 14,000 cps for D2S-C, and 3000 cps for D2S-E.

Discussion

The primary protein sequence in the *D-de* loop region of D2 can be modified drastically without

large effect on the overall performance of PS II. Conserved residues in that region probably serve to optimize PS II properties [6]. The amount of PS II on a chlorophyll basis is reduced in the D2S mutants, indicating adaptation or destabilization of the reaction centers due to the changes in protein composition and/or length in the D2 *D-de* region. This reduction is in agreement with the effect on rates of oxygen evolution at saturating light intensity and on the fluorescence-derived yield of electron transfer [6].

Changes in the binding affinity of PS II-directed herbicides are apparent in most of the mutants from the diuron-binding assay on intact cells (Table I). These changes are generally reflected in the *in vitro* effect of these herbicides on electron flow from water to DCPIP as determined by the herbicide I_{50} values for each of the mutants (Table II). The D2 mutants have a decreased sensitivity to diuron and particularly atrazine, and an increased sensitivity to BNT. The resistances observed are relatively mild compared to some mutations in the D1 protein (such as in the serine 264 change to alanine which confers 100- and 60-fold resistance against diuron and atrazine, respectively [19]), but a number of mutations in D1 lead to herbicide resistance essentially comparable to those seen in the D2 mutants studied in this report [19–21]. The increased sensitivity to BNT found for D2R and for the D2S mutant series was noted also in other D1 mutations leading to diuron resistance [21]. In any case, these similarities suggest a similar mode by which changes in the D1 and D2 proteins modify interactions of at least some herbicides with the Q_B/herbicide-binding environment.

Our results indicate that changes in the *D-de* region of the D2 protein modify (directly or indirectly) the binding environment for some PS II herbicides. This could be interpreted as supporting the concept of a contact point between the *D-de* loops of D1 and D2 [4], but, in view of the many different *D-de* loop sequences yielding very similar herbicide resistance, the specificity of any putative interaction is in doubt. It is possible that the effects on herbicide affinity and efficiency of action result from general changes in the protein environment in the Q_B/herbicide-binding niche, similarly induced by different mutations in D2 and D1. However, irrespective of whether the mutation-induced changes regarding the herbicide-binding niche are direct or not, it is clear that mutations in D2 can affect the properties of the Q_B/herbicide environment significantly.

The thermoluminescence profile of the Q_B peak of the D2S mutants is an additional indication of the involvement of this region of D2 in determining the properties of the Q_B/herbicide-binding environment. Changes in the peak temperature of the Q_B TL curve are indicative of modifications in the Q_B-binding niche. Interestingly, in both D2S-C and D2S-E the TL glow curve has been shifted to higher temperature as compared to wild type, signifying a stabilization of the Q_B semiquinone. D2S-C essentially contains a D1 sequence in the *D-de* region of D2, while in D2S-E the *D-de* loop carries a sequence very different by length and charge from that present in either D1 or D2. The Q_B TL peak of D2S-A is most similar to that found in wild type, even though the D2 *D-de* loop in this mutant has a large charge difference (+12) as compared to wild type.

The very small amplitude of the TL signal seen after one flash in D2S-C and, particularly, in D2S-E indicates that no charge recombination occurs upon heating. Various alterations on the donor side can lead to stabilization of the reducing equivalent on the acceptor side, so that there will be no charge recombination with components on the donor side: for example, if after dark adaptation the water-splitting system is in S_0 state, or the donor D (Tyr-160 in D2) is fully reduced, a reducing equivalent on the acceptor side formed after one flash will be stable. Unless alterations in hydrophilic regions on the cytoplasmic side of D2 alter the properties of the PS II donor side, the most likely mutation-induced modifications would involve the properties of Q_A, the non-heme iron, and/or Q_B. One possible explanation for the results observed is an increase in the semiquinone stability due to an increased number of positive charges close to Q_A and/or Q_B. This would hamper recombination efficiency. Note, however, that the TL amplitude after one flash is also very small in D2S-C, where the overall charge in the *D-de* loop has not been affected significantly. A partially complementary explanation for the lack of charge recombination after one flash is that in D2S-C and D2S-E after dark adaptation essentially 100% of Q_B is in the semiquinone form (as a result

of the stabilization of Q_B^-). After one flash, fully reduced quinol is formed, which is not expected to lead to any TL. A second flash then leads to formation of mostly $S_3 \cdot Q_B^-$, which can recombine leading to TL.

A mutagenesis survey of the *D-de* loop of the D1 protein (H. Kless, M. Oren-Shamir, and M. Edelman, unpublished) indicates that the function of this loop in D1 appears similar to that of D2 in that it modulates herbicide interaction and appears to be involved in rather delicate optimization of PS II performance. The *D-de* loop regions in D1 and D2 may thus be flexible (which perhaps is required for interaction with other polypeptides) rather than rigid as often required for structural roles. These loop regions may thus resemble recognition and binding regions in a variety of proteins, including immunoglobulins and DNA-binding proteins, which are highly flexible [1, 22, 23].

In any case, the results presented here, involving thermoluminescence measurements and herbicide-binding and inhibition experiments, are indicative of significant modifications of the properties of Q_A as well as of the Q_B/herbicide-binding environment in at least some of the D2 mutants. This strengthens the concept of an interaction between D1 and D2 at the cytoplasmic side of the thylakoid, and indicates the importance of the *D-de* loop of D2 in fine-tuning the properties of the acceptor side of PS II.

Acknowledgements

We thank Dr. A. Trebst for providing BNT. This research was funded by a grant of the National Science Foundation (DMB 90-58279) to W. V., a BARD grant to I. O. and W. V., and grants from the EEC and the Israeli Ministry of Science and Technology, and the Forchheimer Center, to M. E. This publication #128 of the Arizona State University Center for the Study of Early Events in Photosynthesis. The Center is funded by DOE grant DE-FG02-88ER13969 as part of the USDA/DOE/NSF Plant Science Centers program.

[1] J. M. Thornton, B. L. Sibanda, and D. J. Barlow, BioEssay **8,** 63–69 (1988).
[2] H. Michel and J. Deisenhofer, Biochemistry **27,** 1–7 (1988).
[3] A. Trebst, Z. Naturforsch. **41c,** 240–245 (1986).
[4] A. Trebst, Z. Naturforsch. **46c,** 557–562 (1991).
[5] B. Svensson, I. Vass, and S. Styring, Z. Naturforsch. **46c,** 765–776 (1991).
[6] H. Kless, W. Vermaas, and M. Edelman, Biochemistry **31,** 11065–11071 (1992).
[7] W. Vermaas, J. G. K. Williams, and C. J. Arntzen, Plant Mol. Biol. **8,** 317–326 (1987).
[8] W. Vermaas, J. Charité, and B. Eggers, in: Current Research in Photosynthesis (M. Baltscheffsky, ed.), **Vol. I,** pp. 231–238, Kluwer Academic Publishers, Dordrecht, The Netherlands 1990.
[9] J. G. K. Williams, Methods Enzymol. **167,** 766–779 (1988).
[10] T. A. Kunkel, J. D. Roberts, and A. Zakour, Methods Enzymol. **154,** 367–382 (1987).
[11] M. A. Vandeyar, M. P. Weiner, C. J. Hutton, and C. A. Batt, Gene **65,** 129–133 (1988).
[12] J. G. K. Williams and D. A. Chisholm, in: Progress in Photosynthesis Research (J. Biggins, ed.), **Vol. IV,** pp. 809–812, Martinus Nijhoff, Dordrecht 1987.
[13] R. Burnap, H. Koike, G. Sotiropoulou, L. A. Sherman, and Y. Inoue, Photosynth. Res. **22,** 123–130 (1989).
[14] W. Vermaas, J. Charité, and G. Shen, Biochemistry **28,** 5325–5332 (1990).
[15] N. Ohad and J. Hirschberg, Photosynth. Res. **23,** 73–79 (1990).
[16] W. Vermaas, J. Charité, and G. Shen, Z. Naturforsch. **45c,** 359–365 (1990).
[17] D. DeVault and Govindjee, Photosynth. Res. **24,** 175–181 (1990).
[18] Y. Inoue, in: The Oxygen-Evolving System (Y. Inoue, A. R. Crofts, Govindjee, N. Murata, G. Renger, and K. Satoh, eds.), pp. 439–450, Academic Press, Tokyo 1983.
[19] N. Ohad and J. Hirschberg, Plant Cell **4,** 273–282 (1992).
[20] A. Horovitz, N. Ohad, and J. Hirschberg, FEBS Lett. **243,** 161–164 (1989).
[21] G. F. Wildner, U. Heisterkamp, and A. Trebst, Z. Naturforsch. **45c,** 1142–1150 (1990).
[22] R. A. Mariuzza, S. E. V. Phillips, and R. J. Poljack, Annu. Rev. Biophys. Chem. **16,** 139–159 (1987).
[23] C. O. Pabo and R. T. Sauer, Annu. Rev. Biochem. **53,** 292–321 (1984).