Biochem. J. (1989) 261, 431–435 (Printed in Great Britain)

Identification of a cysteine residue at the active site of *Escherichia coli* isocitrate lyase

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Escherichia coli isocitrate lyase was inactivated by iodacetate in a pseudo-first-order process. Complete inactivation was associated with the incorporation of only one carboxymethyl group per enzyme subunit. The substrate and products of the enzyme protected against inactivation, suggesting that the reactive group may be located at the active site. Isolation and sequencing of a carboxymethylated peptide showed that the modified residue was a cysteine, in the sequence Cys-Gly-His-Met-Gly-Gly-Lys. The reactivity of isocitrate lyase to iodoacetate declined with pH, following a titration curve for a group of pK_a 7.1. The K_m of the enzyme for isocitrate declined over the same pH range.

INTRODUCTION

During growth of micro-organisms on acetate, the precursors necessary for biosynthesis are generated by carbon flux through the glyoxylate bypass, comprising the enzymes isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) [1]. As a result, there is competition between the glyoxylate bypass and the tricarboxylic acid cycle at the level of isocitrate. In studies of the control of this metabolic branch-point in *Escherichia coli*, considerable attention has been devoted to isocitrate dehydrogenase (EC 1.1.1.42), which is regulated *in vivo* by reversible phosphorylation [2–5]. However, the properties of isocitrate lyase also play an important role in determining the sensitivity of flux control at the branch-point [6].

It is thought that flux through *E. coli* isocitrate lyase is controlled at least partly by the intracellular concentration of isocitrate, which itself is affected by the phosphorylation state of isocitrate dehydrogenase [5–8]. Recently it has been suggested that the activity of isocitrate lyase may be controlled by phosphorylation of the enzyme on a histidine residue [9,10]. The gene that encodes isocitrate lyase, *aceA*, has been cloned and sequenced [11–15]. The enzyme is a tetramer of identical subunits [8], but little is known about its structure, mechanism of action and active site. As part of our wider study of the structure and regulation of *E. coli* isocitrate lyase, we report here on the presence of a reactive cysteine residue at the active site of the enzyme and on the amino acid sequence around this residue.

EXPERIMENTAL

Materials

Iodoacetic acid was from BDH Ltd. Iodo[2-^{3}H]acetic acid was from Amersham International. Acetonitrile and trifluoroacetic acid were from Rathburn Chemicals Ltd. Other chemicals were obtained as described previously [8].

Isocitrate lyase was purified from *E. coli* strain KAT-1/pEM9, which overexpresses the enzymes of the glyoxylate bypass operon of *E. coli* ML308 [11]. The purification procedure was identical to that reported in [8] except that the final step, chromatography on Mono Q, was unnecessary and was omitted. The enzyme was at least 98% pure as judged by polyacrylamide-gel electrophoresis and fast protein liquid chromatography (results not shown).

Assay methods

Isocitrate lyase was assayed by coupling the formation of glyoxylate to the oxidation of NADH by using lactate dehydrogenase as described previously [11]. Unless stated otherwise, the assay buffer was 50 mM-Mops/NaOH, pH 7.3. Measurements of K_m were carried out as in [8]. For experiments at different pH values, 50 mM-Mes/NaOH buffers were used in the range pH 6.0–6.5 and 50 mM-Mops/NaOH buffers in the range pH 6.9–8.1. Protein concentrations were estimated as in [16].

Incubation of isocitrate lyase with iodoacetate

In the standard conditions, isocitrate lyase (1.0 mg/ml) was incubated in 50 mM-Mops/NaOH (pH 7.3)/1 mM-EDTA at 25 °C with 2.0 mM-iodoacetate and other additions as indicated. Samples were assayed for isocitrate lyase activity at various times after the addition of iodoacetate. Half-lives and apparent rate constants were calculated from semilogarithmic plots of activity remaining against time. In some experiments the iodoacetate concentration was varied. In studies of the effect of pH on the reaction, the buffers used were those described above for the assay of isocitrate lyase.

To monitor the extent of carboxymethylation of isocitrate lyase, enzyme was incubated with iodo[2-^{3}H]-acetate (10 mCi/mmol) as described above. Samples were spotted onto 25 mm filter paper discs, washed for 3 × 30 min in 5% (w/v) trichloroacetic acid (30 ml per

Abbreviation used: CMCys, *S*-carboxymethylcysteine.

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disc), rinsed in acetone, dried and counted. Control experiments showed that the washing procedure employed completely removed unbound radioactivity and that the radioactivity incorporated into the protein was stable in these conditions.

To identify the site(s) of modification of isocitrate lyase by iodoacetate, the enzyme (80 nmol of subunits) was incubated with 2 mM-iodo[2-^{3}H]acetate (10 mCi/mmol) at pH 7.3 for 20 min as described above. At this time only 25% of the original activity remained. The reaction was quenched by the addition of dithiothreitol to 15 mM. The protein was freed from unincorporated radioactivity by gel filtration on a column of Sephadex G-25 equilibrated in 0.1 M-Tris/HCl, pH 8.0. It was then reduced with 1 mM-dithiothreitol and carboxymethylated under nitrogen with non-radioactive iodoacetate (10 mM) in the presence of 8 M-urea. The protein was dialysed exhaustively against distilled water and then freeze-dried. It was digested with trypsin (1% by weight for 16 h at 37 °C) and peptides were separated by h.p.l.c. as described in the text.

Determination of amino acid composition and sequence

For amino acid analysis, peptides were hydrolysed for 1 h in 6 M-HCl at 150 °C and analysed using a Waters PICOTAG system. For sequencing, peptides were dissolved in 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid and analysed on an Applied Biosystems 470 gas-phase sequencer equipped with an on-line model 120 PTH-analyser.

RESULTS

Inactivation of isocitrate lyase by iodoacetate

Homogeneous isocitrate lyase was inactivated by iodoacetate in a pseudo-first-order process with a half-life at 2.0 mM-iodoacetate of 8.1 ± 1.0 min (mean ± S.D., $n = 7$) at pH 7.3 and 25 °C (Fig. 1*a*). The apparent pseudo-first-order rate constant for the inactivation, $k'_{app.}$, was directly proportional to the concentration of iodoacetate (Fig. 1*b*), indicating that saturation kinetics do not apply in this case.

The substrate, isocitrate, and the products, glyoxylate and succinate, each protected isocitrate lyase against inactivation by iodoacetate. Representative data for isocitrate are shown in Fig. 2. Previous work has shown that the substrate for isocitrate lyase is D$_s$-isocitrate and that L$_s$-isocitrate is neither a substrate nor an inhibitor of the enzyme [8 and references cited therein]. Consistent with this, it was found that 200 μM-D$_s$-isocitrate afforded the same degree of protection against inactivation as 400 μM-DL-isocitrate (results not shown). Values of K_s were determined from replots of half-life against effector concentration (e.g. Fig. 2). The values for D$_s$-isocitrate, glyoxylate and succinate were 0.095, 0.31 and 0.80 mM respectively. These values should be compared to the kinetically determined K_m values of 0.063, 0.13 and 0.59 mM respectively [8]. It should be noted that the values for K_s were determined in the absence of the essential cofactor Mg^{2+}, and that the enzyme obeys an equilibrium kinetic mechanism [8]. The fact that the substrate and products of the enzyme afforded protection against iodoacetate, and the similarity between the K_s and K_m values, suggest strongly that iodoacetate modifies a group at the active site of isocitrate lyase.

Stoichiometry and site of modification by iodoacetate

The incorporation of iodo[2-^{3}H]acetic acid into isocitrate lyase was measured as described in the Experimental section. The results of several experiments (Fig. 3) show that the extent of inactivation of the enzyme correlated with the incorporation of radioactivity. Complete inactivation corresponded to the incorporation of just over one carboxymethyl group per enzyme subunit (Fig. 3). Further incubation beyond this point resulted in very little additional incorporation of radioactivity into

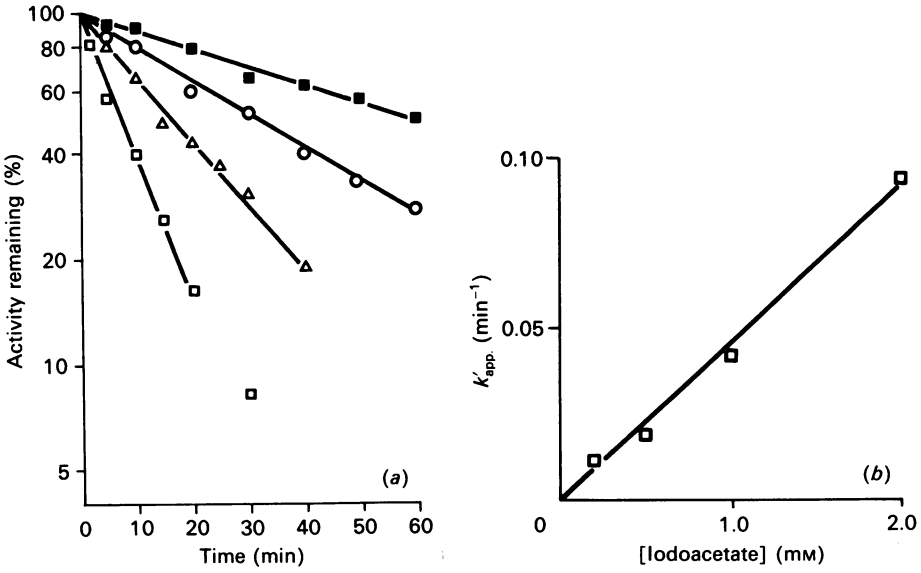

Fig. 1. Inactivation of isocitrate lyase by iodoacetate

(*a*) Isocitrate lyase was incubated as described in the Experimental section in the presence of 0.2 mM (■), 0.5 mM (○), 1.0 mM (△) or 2.0 mM (□) iodoacetate. (*b*) The apparent pseudo-first-order rate constant for the inactivation, $k'_{app.}$, was plotted against iodoacetate concentration.

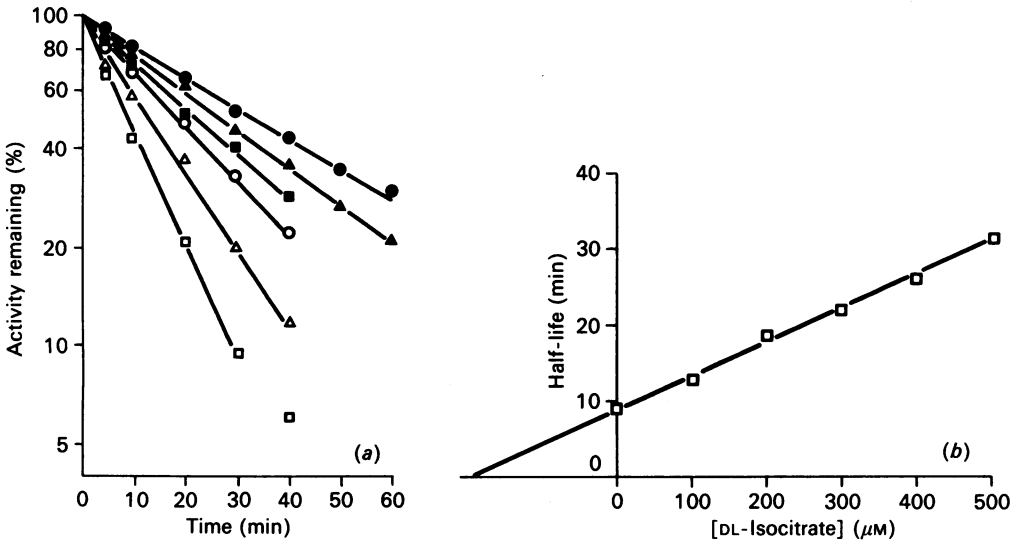

Fig. 2. Protection by isocitrate against inactivation by iodoacetate

(*a*) Isocitrate lyase was incubated in the presence of 2.0 mM-iodoacetate and the following concentrations of DL-isocitrate: 0 (□), 100 μM (△), 200 μM (○), 300 μM (■), 400 μM (▲) or 500 μM (●). (*b*) The half-life of the enzyme was plotted against the concentration of DL-isocitrate. The concentration of the biologically active isomer, D_s-isocitrate, is taken as exactly half that of DL-isocitrate [8].

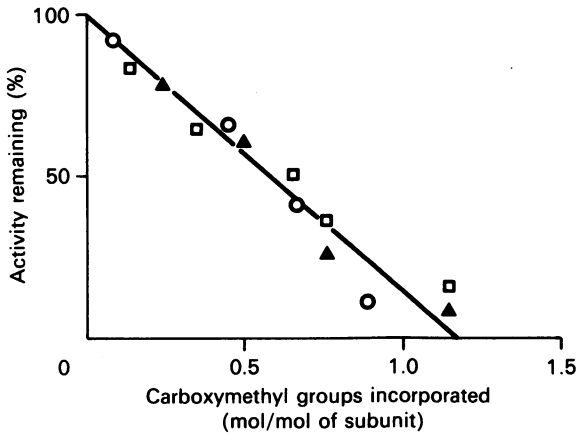

Fig. 3. Stoichiometry of modification by iodacetate

Incorporation of iodo[2-^{3}H]acetate into isocitrate lyase was measured as described in the Experimental section. The different symbols show data from three independent experiments.

the enzyme (results not shown). These results indicate that the active site of isocitrate lyase contains one group that is particularly reactive to iodoacetate.

The labelling of isocitrate lyase was scaled up and the protein was digested with trypsin as described in the Experimental section. The peptide mixture was first fractionated using a Waters C_{18} reverse-phase column. This yielded a single major radioactive peak, containing greater than 90% of the recovered ^{3}H, that was retarded by but not bound to the column in 0.1% (v/v) trifluoroacetic acid. Most of the peptide material was bound to the column in these conditions. The radioactive peak was further purified by gel filtration on a TSK 2000GW column (7.5 mm internal diameter × 600 mm; Anachem Ltd., Luton, Beds., U.K.). This procedure gave a single peak of radioactivity which coincided with a peak of absorbance at 215 nm. However, sequencing of this material revealed the presence of at least two distinct peptides. Finally, the peptide was re-purified by reverse-phase chromatography on a Waters C_{18} column. As shown in Fig. 4, the material bound to the column in 0.1% trifluoroacetic acid, and was eluted with an increasing gradient of acetonitrile concentration. The difference in the chromatographic behaviour of the peptide between this experiment and the use of reverse-phase chromatography earlier in the purification was apparently due to use of a newer column in this case (similar observations to this effect have been made independently in both laboratories contributing to this work).

In this experiment (Fig. 4) two radioactive peaks were observed. Peak 1, the earlier-eluting peak, contained some 25% of the total recovered ^{3}H. This peak had the composition CMCys 1.0, Gly 3.3, His 0.9, Met 1.0, Lys 0.9. The composition of the major peak was similar except that methionine was replaced by methionine sulphoxide (results not shown). These results show that the two radioactive peaks observed in Fig. 4 are derived from the same peptide by partial oxidation of a methionine residue, which probably occurred during storage. Gas-phase sequencing of a sample of peak 2 showed the sequence to be as follows, with the amount of each residue detected given in parentheses: CMCys (35 pmol); Gly (32 pmol); His (22 pmol); Met (28 pmol); Gly (32 pmol); Gly (25 pmol); Lys (24 pmol).

The methionine sulphoxide in peak 2 was presumably reduced to methionine by the high concentration of dithiothreitol present during Edman degradation.

Effect of pH on isocitrate lyase

The value of $k'_{app.}$, the apparent pseudo-first-order rate constant for the inactivation of isocitrate lyase by iodoacetate, increased more than 6-fold between pH 6.1 and 8.1. Assuming that the protonated form of the enzyme is

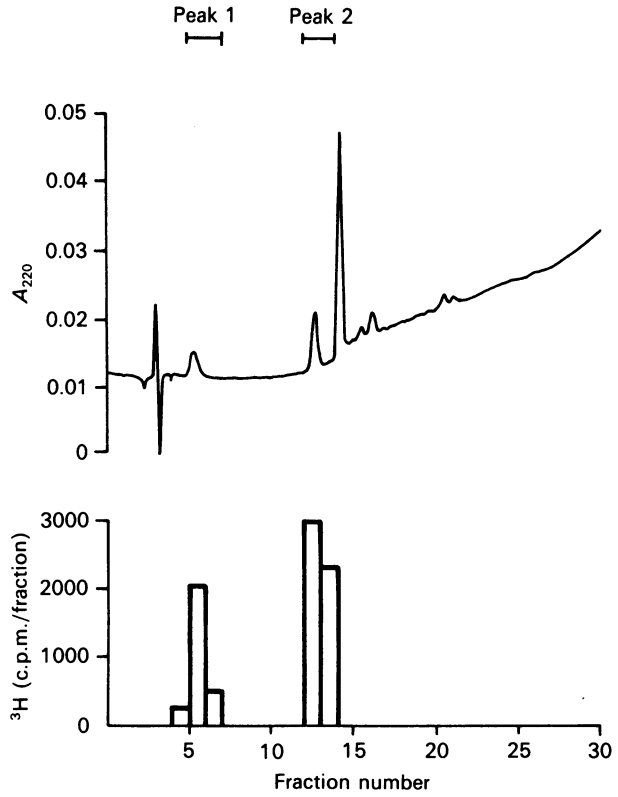

Fig. 4. Purification of a carboxymethylated peptide from isocitrate lyase

A sample (approx. 1 nmol) of the peptide purified by reverse-phase and gel filtration chromatography (see text) was loaded onto a Waters μBondapak C_{18} reverse-phase column equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with a linear gradient of 0–50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid run over 30 min at a flow rate of 1 ml/min. A_{220} was monitored continuously and 1 ml fractions were collected. The bars indicate the fractions pooled as peaks 1 and 2 (see the text).

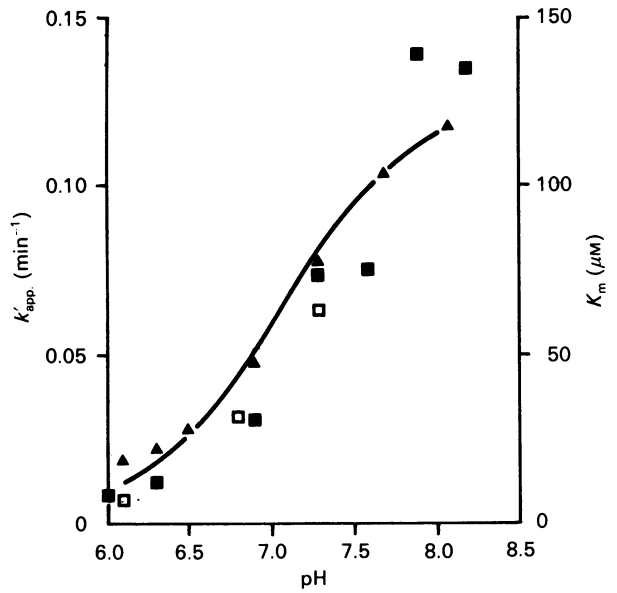

Fig. 5. Effect of pH on the reactivity of isocitrate lyase to iodoacetate and its K_m for isocitrate

Isocitrate lyase was incubated with iodoacetate at different pH values as described in the Experimental section. Pseudo-first order rate constants ($k'_{app.}$) were obtained as in Fig. 2 (▲). K_m values for D_s-isocitrate were obtained in this study (■) or are taken from [8] (□). The solid line is a theoretical titration curve assuming extreme values of $k'_{app.}$ of 0 and 0.13 min^{-1}, and a pK_a of 7.1.

unreactive and that the value of $k'_{app.}$ for the fully unprotonated form is 0.130 min^{-1}, the data fit a titration curve for a group of pK_a 7.1 reasonably well (Fig. 5). The K_m of isocitrate lyase for isocitrate also varies markedly over this pH range (Fig. 5). However, it is difficult to make a detailed analysis of this behaviour because isocitrate itself ionizes in the lower part of this pH range, with its third pK_a being 6.40 [17].

DISCUSSION

Previous studies, involving modification with bromopyruvate, have implicated a cysteine residue at the active site of isocitrate lyase. For the enzyme from *Neurospora crassa*, *Pseudomonas indigofera* and watermelon cotyledons, bromopyruvate reacted with a single cysteine residue per subunit and gave complete inactivation in a process that showed saturation kinetics [18–20]. However the sequence surrounding the reactive cysteine residue has not been reported. Substrate protection data suggested that in the enzyme from *P. indigofera*, the cysteine residue was part of the succinate subsite [19], whereas in the enzyme from *N. crassa* and *Linum usitatissimum* (flax), it was part of the glyoxylate subsite [18,21]. Nevertheless, the results suggested that bromopyruvate acts as an affinity labelling reagent for isocitrate lyase. This work led to a proposed catalytic mechanism for the enzyme in which the cysteine residue donates a proton to the C-3 atom of isocitrate during cleavage [19].

The data reported here show that carboxymethylation of a single cysteine residue completely inactivates *E. coli* isocitrate lyase. The protection afforded by substrates strongly suggests that, as with the enzyme from other sources, this cysteine residue is at the active site of the enzyme, but it cannot be assigned specifically to the glyoxylate or succinate subsite. Unlike bromopyruvate, iodoacetate does not act as an affinity labelling reagent for isocitrate lyase. This implies that, of the five cysteine residues in *E. coli* isocitrate lyase [15], only one, that at the active site, is particularly reactive towards iodoacetate.

The sequence containing this presumed active site cysteine residue, Cys-Gly-His-Met-Gly-Gly-Lys, agrees exactly with residues 195–201 of the amino acid sequence deduced from the nucleotide sequence of the *aceA* gene of *E. coli* K12 [14,15]. A very similar sequence, Cys-Gly-His-Met-Gly-Ala-Lys, is found within the deduced amino acid sequence of the isocitrate lyase of castor bean [22]. Matsuoka & McFadden [15] recently reported 39% identity between the deduced amino acid sequences of the *E. coli* and castor bean enzymes. The active-site cysteine residue occurs within a region that is particularly highly conserved; comparison of residues 173–243 of the *E. coli* sequence [14,15] with residues 191–261 of the castor bean sequence [22] reveal 47 identities and eight

conservative changes. This level of similarity might suggest that the two enzymes have structurally similar catalytic domains and similar mechanisms. In this context, Malhotra *et al.* [23] recently reported some surprising results on the isocitrate lyase from castor bean. This enzyme contained an active-site cysteine residue, located at the glyoxylate subsite, as judged by inactivation experiments with *N*-ethylmaleimide. Iodoacetate also inactivated this enzyme, but without affecting cysteine residues; the authors favoured the view that iodoacetate reacted with an active-site histidine residue [32]. We detected no carboxymethylated histidine. It is possible that, in spite of the sequence similarities, the disposition and reactivity of groups within the active site differs between the two enzymes.

The rate of inactivation of *E. coli* isocitrate lyase by iodoacetate was markedly reduced below pH 7. Assuming that the protonated form of the enzyme is essentially unreactive, the data (Fig. 5) correspond to the titration curve of a single ionizing group with a pK_a of 7.1. The identity of this group is not known. It might be a group, spatially distinct from the active site, whose protonation results in a conformational change that renders the cysteine residue inaccessible to iodoacetate; this explanation cannot be ruled out, even though c.d. studies revealed no detectable changes in the conformation of the enzyme between pH 6.5 and 7.5 (results not shown). It might be another group in the active site whose protonation affects both the binding of isocitrate and the accessibility of the cysteine residue to iodoacetate. It might even be the cysteine residue itself, although the pK_a of 7.1 is some two pH units below the expected value [24]. This possibility is consistent with the data of Lindley [25] on the reaction of mercaptoacetic acid with chloracetamide which suggest that the RS^- species would be much more reactive to iodoacetate than would the RSH species. More detailed structural studies will be required to test these possibilities.

This work was supported by grants from the Medical Research Council and the Science and Engineering Research Council.

Received 6 January 1989/1 March 1989; accepted 8 March 1989

REFERENCES

1. Kornberg, H. L. (1966) Essays Biochem. **2**, 1–31
2. Bennett, P. M. & Holms, W. H. (1975) J. Gen. Microbiol. **87**, 37–51.
3. Garnak, M. & Reeves, H. C. (1979) Science **203**, 1111–1112
4. Borthwick, A. C., Holms, W. H. & Nimmo, H. G. (1984) Biochem. J. **222**, 797–804
5. Walsh, K. & Koshland, D. E., Jr. (1985) J. Biol. Chem. **260**, 8430–8436
6. LaPorte, D. C., Walsh, K. & Koshland, D. E., Jr. (1984) J. Biol. Chem. **259**, 14068–14075
7. El-Mansi, E. M. T., Nimmo, H. G. & Holms, W. H. (1985) FEBS Lett. **183**, 251–255
8. MacKintosh, C. & Nimmo, H. G. (1988) Biochem. J. **250**, 25–31
9. Robertson, E. F., Hoyt, J. C. & Reeves, H. C. (1988) J. Biol. Chem **263**, 2477–2482
10. Hoyt, J. C. & Reeves, H. C. (1988) Biochem. Biophys. Res. Commun. **153**, 875–880
11. El-Mansi, E. M. T., MacKintosh, C., Duncan, K., Holms, W. H. & Nimmo, H. G. (1987) Biochem. J. **242**, 661–665
12. Chung, T., Klumpp, D. J. & LaPorte, D. C. (1988) J. Bacteriol. **170**, 386–392
13. Cortay, J. C., Bleicher, F., Rieul, C., Reeves, H. C. & Cozzone, A. J. (1988) J. Bacteriol. **170**, 89–97
14. Rieul, C., Bleicher, F., Duclose, B., Cortay, J. C. & Cozzone, A. J. (1988) Nucleic Acids Res. **16**, 5689
15. Matsuoka, M. & McFadden, B. A. (1988) J. Bacteriol. **170**, 4528–4536
16. Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
17. Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969) Data for Biochemical Research, 2nd ed., pp. 74–75, Oxford University Press, Oxford
18. Johanson, R. A., Hill, J. M. & McFadden, B. A. (1974) Biochim. Biophys. Acta **364**, 341–352
19. Roche, T. E., McFadden, B. A. & Williams, J. O. (1971) Arch. Biochem. Biophys. **147**, 192–200
20. Jameel, S., El-Gul, T. & McFadden, B. A. (1985) Arch. Biochem. Biophys. **236**, 72–81
21. Khan, F. R. & McFadden, B. A. (1982) Plant Physiol. **70**, 943–948
22. Beeching, J. R. & Northcote, D. H. (1987) Plant Mol. Biol. **8**, 471–475
23. Malholtra, O. P., Dwivedi, U. N., Singh, J. & Srivastava, P. K. (1987) Ind. J. Biochem. Biophys. **24**, 57–62
24. Tanford, C. (1962) Adv. Protein Chem. **17**, 69–165
25. Lindley, H. (1960) Biochem. J. **74**, 577–584