The conformational flexibility and long-range interactions in rabbit muscle aldolase induced by active-site ligand binding, cross-linking of the enzyme between Cys\textsuperscript{72} and Cys\textsuperscript{338}, and removal of the C-terminal tyrosine residue were studied by following the changes in the microenvironments of Cys\textsuperscript{239} and Cys\textsuperscript{240} located outside the active site. It was found that substrates induced a conformational change in aldolase, which propagates from the active site to Cys\textsuperscript{239}, which is located close to intersubunit contacts. The response of the enzyme is differential. Ligands having both C-1 and C-6 phosphates or C-1 phosphate only induce the enhancement of Cys\textsuperscript{239} reactivity, whereas those with C-6 phosphates only decrease Cys\textsuperscript{239} reactivity. This correlates well with a dramatic difference in kinetic parameters for a cleavage of fructose-1,6-P\textsubscript{2} and fructose-1-P. Therefore, these changes can be interpreted as syncatalytic.

Cross-linking of the aldolase subunit by an \(-\text{S-S-}\) bridge between Cys\textsuperscript{72} and Cys\textsuperscript{338} inactivates the enzyme, abolishes binding of active-site ligands, and induces a conformational change in the enzyme that can be detected far away (at Cys\textsuperscript{239} and Cys\textsuperscript{240}) from the site of perturbation. Cys\textsuperscript{72} and Cys\textsuperscript{338} are not in the active site. This shows that the region of the active site and the environment of Cys\textsuperscript{72} and Cys\textsuperscript{338} are tightly coupled and that residues far away from the active site, through such coupling, can possess properties of active-site residues. Similar, although less dramatic changes are observed upon removal of the C-terminal tyrosine residue.

In view of the results obtained in this paper, aldolase seems to be quite a flexible molecule, whose conformation is sensitive to the nature of a substrate bound to the enzyme and is able to transmit the information about a local perturbation over long distances within a molecule.

Long-range effects and substrate-induced conformational flexibility in aldolase were probed in this paper by measuring the changes in the chemical reactivity of Cys\textsuperscript{239} and Cys\textsuperscript{240} in response to perturbation of protein by substrate binding, removal of the C-terminal tyrosine, and formation of the intrasubunit \(-\text{S-S-}\) bond between Cys\textsuperscript{72} and Cys\textsuperscript{338}. The sites of perturbation of the enzyme molecule are at considerable distance from Cys\textsuperscript{239} and Cys\textsuperscript{240}, therefore, the changes in their reactivity in response to these perturbations can be interpreted as evidence for long-range effects. The obtained results show that aldolase is quite a flexible molecule, able to transmit the information about local perturbation to distant parts of the molecule. Substrate-induced perturbations are syn-catalytic in nature. Substrates having only C-1 phosphate induce a different conformational change compared with those having both C-1 and C-6 phosphates. This may explain the dramatic difference in kinetic properties between fructose-1-P and fructose-1,6-P\textsubscript{2}.

The classical approach to identify active-site residues in enzymes is to study the inactivation of the enzyme by chemical modification and protection against inactivation by substrate binding. The modern version of this approach would be to employ site-directed mutagenesis to study the effect of amino acid substitutions on enzyme catalytic properties. Interpretation of the results of these experiments frequently relies on the assumption that long-range effects are unimportant, i.e. for example, perturbation of the enzyme caused by substitution or modification of a residue distant from the active site is not transmitted to the active-site region. In many instances, this assumption is correct, but probably not always.

In aldolase A, Cys\textsuperscript{72} and Cys\textsuperscript{338}, which are close to each other (Kobashi and Horecker, 1967; Sygusch \etal, 1987), are protected by substrate against chemical modification (Steinman and Richards, 1970); and it was believed that they are essential for catalysis (Lai \etal, 1974). Later, based on chemical modification studies and site-directed mutagenesis (Heyduk and Kochman, 1986; Takahashi \etal, 1989), these cysteines were shown to be nonessential. They also appear to be quite distant from the active site as evident from the crystallographic structure of the enzyme (Sygusch \etal, 1987). Therefore, one may conclude that there is long-range communication between the active-site region and the microenvironment of Cys\textsuperscript{72} and Cys\textsuperscript{338}.

The molecule of rabbit muscle aldolase consists of four identical or nearly identical subunits with an M\textsubscript{r} of 40,000 (Kawahara and Tanford, 1966; Penhoet \etal, 1967; Susor \etal, 1969). Each subunit of the enzyme contains four buried and four exposed sulfhydryl groups (Lai \etal, 1974; Steinman and Richards, 1970; Anderson and Perham, 1970). One of the exposed thiol groups (Cys\textsuperscript{239}) exhibits high reactivity toward various thiol reagents; the remaining three groups react much more slowly (Steinman and Richards, 1970). The difference in the kinetics of the chemical modification of these cysteines is so large that it is easy to monitor their reactivity independently (Eagles \etal, 1969; Anderson and Perham, 1970; Heyduk and Kochman, 1985).

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EXPERIMENTAL PROCEDURES

Materials—Hexitol-1,6-P\textsubscript{2} was prepared by the reduction of Fru-1,6-P\textsubscript{2} with sodium borohydride (Ginsburg and Mehler, 1966). Nbs\textsubscript{2} \textsuperscript{1}

\textsuperscript{1} The abbreviations used are: Nbs\textsubscript{2}, 5,5'-dithiobis(2-nitrobenzoic
Kinetic Measurements—A Spectro M-40 spectrophotometer was used for the investigation of the kinetics of Nbs release from the aldolase-Cys 23'-Nbs derivative. The instrument was equipped with a kinetic data acquisition and analysis accessory. The reaction was started by addition of 50 µl of 20 mM glutathione to a cuvette containing 2 ml of the 4.8 mM Nbs2 solution. The progress of the reaction was monitored at 412 nm. For each curve, ~300 data points were usually collected, and pseudo first-order rate constants (k) were obtained by nonlinear regression analysis of the collected data. The reaction obeys pseudo first-order kinetics at least up to 95% of completion.

Effect of Ligands on Reactivity of Sulphydryl Groups—The results of kinetic studies on the modification and demodification of Cys 23' in the presence of various active-site ligands shown as ratios (R) of rate constants measured in the presence and absence of ligands are shown in Table I. Although the presented results for demodification are for experiments performed with glutathione, the same results (R values) were obtained with different thiols (2-mercaptoethanol, cysteine, dithiothreitol) and also when Cys 23' was modified with 2,2'-bipyridyl.
concentration is low, the products of the reaction (DHAP and glyceraldehyde 3-phosphate). The latter decreases the value, whereas those having only a C-6 phosphate or both C-1 and C-6 phosphates decreased the value for demodification is clearly lower. The pattern of the changes in Cys239 reactivity seems to be a function of the distribution of phosphate groups in the ligand molecule. Active-site ligands possessing a phosphate moiety bound only to the C-1 phosphate-binding site on the enzyme increase the R value, whereas those having only a C-6 phosphate or both C-1 and C-6 phosphates decreased the R value or caused no change at all. There seems to be an apparent discrepancy between the effects of Fru-1,6-P2 and hexitol-1,6-P2 since the former results in an R value of essentially 1, whereas the latter decreases the R value. This can be explained if one remembers that mixing aldolase with Fru-1,6-P2 results in an equilibrium mixture of Fru-1,6-P2 and phosphotrioses (DHAP and glyceraldehyde-3-phosphate). The R value in the presence of Fru-1,6-P2 is therefore averaged over all the different complexes present in solution. To illustrate this, we have taken advantage of the high dependence of the percentage of Fru-1,6-P2 in the equilibrium mixture on the starting concentration of this substrate (Fig. 1). When the Fru-1,6-P2 concentration is low, the products of the reaction (DHAP and glyceraldehyde-3-P) prevail, and the aldolase-DHAP complex should be a major species among different enzyme-substrate complexes. Therefore, the R value is 1 (Fig. 1). When the starting concentration of Fru-1,6-P2 is high (and therefore percent of this substrate in the equilibrium mixture), the R value decreases to a value of <1, as found for hexitol-1,6-P2. So, we conclude that Fru-1,6-P2 has an effect on the reactivity of Cys239 similar to hexitol-1,6-P2.

The changes of the reactivity of Cys239 observed upon ligand binding (measured by both the rate of modification and demodification of this residue) display saturation kinetics when studied as a function of ligand concentration. This is illustrated in Fig. 2 for DHAP. The dissociation constant for DHAP calculated from these data by nonlinear regression for the equation describing binding of ligand to four independent sites is 4.9 μM. This is in agreement with the values obtained by different methods (Grazi and Trombetta, 1974). Therefore, it can be concluded that the measured changes in thiol reactivities are the result of formation of the specific enzyme-ligand complex.

The reactivity of the second exposed thiol group in aldolase, Cys389, was also measured. In contrast to the results for Cys239, active-site ligands did not change the reactivity of Cys389 (data not shown). These results indicate that the region of the aldolase molecule comprising the environment of Cys389 is not affected by ligand binding.

**Fluorescence quenching of aldolase-Cys239-EDANS derivative**—The fluorescence of the aldolase-Cys239-EDANS derivative (both intensity and λmax) is not changed significantly upon binding of active-site ligands (data not shown). However, changes in the microenvironment of Cys239 can be seen if fluorescence quenching of aldolase-Cys239-EDANS is studied.

The quenching curves for both acrylamide and KI are nonlinear (Fig. 3, A and B). A curvature of this type in Stern-Volmer plots can in general be caused either by a heterogenous population of fluorophores or by poor quenching efficiency of a quencher (Eftink and Ghiron, 1981). This latter possibility should be discarded since the plots for the cysteine-EDANS

### Table 1

| Ligand            | Modification of Cys239 | Modification of Cys389 |
|-------------------|-----------------------|-----------------------|
| None              | 1.00                  | 1.00                  |
| DHAP              | 1.36 ± 0.05           | 1.25 ± 0.04           |
| Hexitol-1,6-P2    | 0.73 ± 0.03           | 0.71 ± 0.02           |
| Fru-1,6-P2        | 1.03 ± 0.03           | 1.04 ± 0.05           |
| Fru-1-P           | 1.24 ± 0.05           | 1.29 ± 0.04           |
| Fru-6-P           | 0.95 ± 0.06           | 0.93 ± 0.04           |
| P1                | 1.60 ± 0.06           | 1.23 ± 0.05           |
| β-Glycerophosphate| 1.23 ± 0.01           |

**Fig. 1.** Dependence of R for demodification of aldolase-Cys239-Nbs by glutathione on Fru-1,6-P2 concentration. Demodification was performed at various concentrations of Fru-1,6-P2; and for each concentration, the R value was established. The percent of Fru-1,6-P2 in the equilibrium mixture was calculated assuming the equilibrium constant for the aldolase reaction to be K = 0.3 × 10^{-5} M.

**Fig. 2.** Dependence of R for aldolase-Cys239-Nbs demodification by glutathione on DHAP concentration. The solid line represents the best fit to the data obtained by nonlinear regression. The data were fitted to the equation describing binding of ligand to four independent sites on the enzyme (Kasprzak and Kochman, 1980). The best fit was obtained for Kd = 4.9 μM.
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judged by correlation matrix analysis (Johnson and Frazier, 1985). Therefore, to compare the accessibility of the fluorophores to quenching, the apparent quenching constants were used that were calculated as weight averages of fitted parameters, i.e. 

\[ K_{app} = f_1K_1 + f_2K_2 \]  

(II). Since aldolase-Cys\(^\text{329}\)-EDANS is a dominant fraction of the fluorophores, \( K_{app} \) mostly reflects the accessibility of this fluorophore.

The results obtained show that in the absence of substrates, Cys\(^\text{329}\)-EDANS is somewhat shielded from the solvent since the \( K_{app} \) value is less than the one for cysteine-EDANS. The addition of hexitol-1,6-P\(_2\) or DHAP increases the accessibility of this fluorophore (\( K_{app} \) increases). In the case of KI quenching, Cys\(^\text{329}\)-EDANS in the absence of ligands is more quenched than cysteine-EDANS. This shows that there is some favorable distribution of positive charges around the fluorophore in Cys\(^\text{329}\)-EDANS that makes negatively charged I\(^-\) so effective. In the case of KI, contrary to acrylamide quenching, addition of substrates dramatically decreases \( K_{app} \) values.

In the presence of 6 M guanidine HCl, all these effects of ligands disappear, i.e. quenching curves with or without ligands are the same (Fig. 3, A and B).

**Effect of Cross-linking of Aldolase on Reactivity of Thiol Groups**—Cross-linking of aldolase by the intrasubunit -S-S- bond between Cys\(^72\) and Cys\(^338\) results in the increase of reactivity of the fast-reacting Cys\(^\text{329}\) residue and of the slowly reacting Cys\(^\text{360}\) residue (Table III). No effect of hexitol-1,6-P\(_2\) on the reactivity of Cys\(^\text{329}\) or Cys\(^\text{390}\) was observed. In this aldolase derivative, fluorescence titration showed that cross-linked aldolase is unable to bind DHAP, hexitol-1,6-P\(_2\), ATP, or P\(_\text{i}\) (Table IV). Cross-linked aldolase after reduction with 0.5 mM dithiothreitol, 100 mM Tris, 1 mM EDTA, pH 7.5, for 160 min recovers 97% of the native enzyme activity and the ability of binding active-site ligands, indicating that cross-linking does not cause irreversible change in aldolase structure.

### Table II

| Quencher | Sample                      | \( K_{app} \) |
|----------|-----------------------------|---------------|
|          |                             | \( M^{-1} \)  |
| Acrylamide | Aldolase                      | 5.24          |
|          | Aldolase + hexitol-1,6-P\(_2\) | 5.59          |
|          | Aldolase + DHAP               | 6.07          |
|          | Aldolase in 6 M GdnHCl \pm ligands | 2.13        |
|          | Cysteine-EDANS               | 7.93          |
|          | Cysteine-EDANS in 6 M GdnHCl | 2.39          |
| KI       | Aldolase                      | 2.39          |
|          | Aldolase + hexitol-1,6-P\(_2\) | 0.61          |
|          | Aldolase + DHAP               | 0.34          |
|          | Aldolase in 6 M GdnHCl \pm ligands | 0.28        |
|          | Cysteine-EDANS               | 0.24          |
|          | Cysteine-EDANS + 6 M GdnHCl  | 0.13          |

* GdnHCl, guanidine hydrochloride.

### Table III

| Aldolase | Modification of Cys\(^329\) | Modification of Cys\(^390\) |
|----------|-----------------------------|-----------------------------|
| Native   | 1.00                        | 1.00                        |
| Cross-linked | 1.41 \pm 0.06             | 1.71 \pm 0.07             |
| Cross-linked + hexitol-1,6-P\(_2\) | 1.41 \pm 0.06 | 1.70 \pm 0.07 |
| Des-Tyr\(^361\) | 1.45 \pm 0.05 | 1.19 \pm 0.05 |
| Des-Tyr\(^329\) and cross-linked | 3.15 \pm 0.09 | 2.06 \pm 0.07 |

**Effect of cross-linking and carboxypeptidase digestion on the reactivity of thiol groups in aldolase.**

The rates of modification of Cys\(^\text{329}\) and Cys\(^\text{390}\) were measured in native, cross-linked, and carboxypeptidase-degraded aldolase; and for each enzyme, \( R \) values were obtained.

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**Fig. 3.** Stern-Volmer plot for quenching of aldolase-Cys\(^\text{329}\)-EDANS fluorescence in presence or absence of ligands. A, acrylamide quenching; B, KI quenching. ○, no ligands added; ●, ± 1 mM hexitol-1,6-P\(_2\); ●, ± 1 mM hexitol-1,6-P\(_2\) or cysteine-EDANS; filled symbols, ± ligands in presence of 6 M guanidine HCl. Fluorescence was excited at 340 nm and observed at 490 nm. Solid lines represent the best fit to the Stern-Volmer equation as described in text.

The simplest scheme that gave satisfactory fit for all curves (Fig. 3, A and B) was the one with \( n = 2 \) and with no static quenching (\( V_i = 0 \)). For all curves, the distribution of fluorophores was 80–90% of one kind and 10–20% of the other kind. It is reasonable to ascribe the fluorophore that is prevailing to the Stern-Volmer equation as described in text. Therefore, quenching curves were fitted to a general equation describing quenching of \( n \) independent fluorophores:

\[ F/F_0 = \sum_i f_i(1 + K_i[Q])\exp(V_i[Q]), \]

where \( V_i \) is a static quenching constant and \( Q \) is a concentration of a quencher.
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**TABLE IV**

Parameters of ligand binding to native aldolase and its derivatives

| Ligand    | Native aldolase | Des-Tyr<sup>363</sup>-aldolase | Cross-linked aldolase |
|-----------|-----------------|---------------------------------|-----------------------|
| DHAP      | \( n = 4.1 \pm 0.1 \) | \( K_d = 10.3 \pm 0.3 \) \( \mu \text{M} \) | \( n = 2.9 \pm 0.4 \) | ND<sup>a</sup> |
| Hexitol-1,6-P<sub>2</sub> | \( n = 4.3 \pm 0.1 \) | \( K_d = 2.6 \pm 0.5 \) \( \mu \text{M} \) | \( n = 4.1 \pm 0.1 \) | ND |
| ATP       | \( n = 4.5 \pm 0.5 \) | \( K_d = 34.0 \pm 4.0 \) \( \mu \text{M} \) | ND                        | ND |
| P<sub>i</sub> | \( n = 4.0 \) | \( K_d = 780 \pm 30 \) \( \mu \text{M} \) | ND |

<sup>a</sup> ND, not detected.

<sup>b</sup> Because of a high dissociation constant, the number of binding sites could not be obtained. The value of \( K_d \) was obtained assuming \( n = 4.0 \).

The Reactivity of Cys<sup>39</sup> and Cys<sup>289</sup> in Des-Tyr<sup>363</sup>-aldolase—The des-Tyr<sup>363</sup>-aldolase derivative has about four binding sites for hexitol-1,6-P<sub>2</sub> and DHAP and exhibits decreased affinities for these ligands as compared to the native enzyme (Table IV). As shown in Table III, removal of Tyr<sup>363</sup> results in a large increase in the reactivity of Cys<sup>289</sup> and a substantial increase in the reactivity of Cys<sup>39</sup>. Because the change in the reactivity of Cys<sup>289</sup> in des-Tyr<sup>363</sup>-aldolase was almost identical to that observed in the cross-linked enzyme, the reactivity of cysteine residues was investigated in cross-linked des-Tyr<sup>363</sup>-aldolase. As shown in Table III, cross-linking of des-Tyr<sup>363</sup>-aldolase via the Cys<sup>72</sup>-Cys<sup>338</sup> disulfide bridge results in a further increase in the reactivity of both Cys<sup>39</sup> and Cys<sup>289</sup> residues. This indicates that conformation changes induced by removal of the C-terminal Tyr<sup>363</sup> residue are distinct from those induced by cross-linking of aldolase.

**DISCUSSION**

**Syncatalytic Nature of Substrate-induced Conformational Changes in Aldolase A**—The reactivity of thiol groups was successfully used in the detection of temperature-induced conformational transition in the aldolase molecule (Heyduk and Kochman, 1985). In this study, this technique was applied to the investigation of conformational changes upon binding of active-site ligands. Conformational changes induced by ligand binding have already been proposed for aldolase. These suggestions were based on experiments with proteolysis in the presence of substrates (Adelman et al., 1968) and on differences in UV spectrum after binding of arabinofuranosyl-1,5-bisphosphate, a strong inhibitor of aldolase (Crowder et al., 1973). However, there are interesting features of these changes that become apparent from this work.

All reactive cysteine residues of aldolase are localized outside the active site of the enzyme (Sygusch et al., 1987) (also see Fig. 4). Conformational changes induced by active-site ligand binding can be detected at Cys<sup>289</sup> (by cysteine reactivity and fluorescence measurements) and at Cys<sup>72</sup>-Cys<sup>338</sup> (by cysteine reactivity (Steinman and Richards, 1970)) and are undetectable at Cys<sup>289</sup>. This shows that perturbation of the enzyme conformation by substrate binding is transmitted from the active site to remote parts of a molecule (see Fig. 4). It also seems that it is not a global rearrangement of the protein structure since some regions (e.g., Cys<sup>289</sup>) are unaffected by substrate binding. Cys<sup>289</sup> is positioned close to the intersubunit contact region (Sygusch et al., 1987) (Fig. 4), indicating that the signal of substrate binding can be transmitted to another subunit (it does not necessarily mean that it is transmitted to the second active site). Aldolase A is not an allosteric enzyme and displays pure Michaelis-Menten kinetics for substrate binding (Penhoet et al., 1969; Horecker et al., 1972). Other active-site ligands also seem to bind to the enzyme in a noncooperative mode (Kaspzak and Kochman, 1980; Palczewski et al., 1983, 1995; Palczewski and Kochman, 1987). On the other hand, binding of inositol polyphosphates to aldolase A is sigmoidal (Koppitz et al., 1984). Hybridization studies between aldolase A<sub>4</sub> and C<sub>4</sub> show that antibody binding to C-type subunits can inhibit enzyme activity by as much as 60% in A<sub>C</sub> tetramer (a hybrid composed of three subunits of aldolase A and one of aldolase C) (Penhoet and Rutter, 1971). The removal of C-terminal tyrosine residues from lobster or snail muscle aldolase with carboxypeptidase is much slower than the loss of activity of these enzymes (Guha et al., 1971; Buczylik et al., 1980). All of the above can be interpreted as the existence of cooperative interactions between aldolase subunits.

The most interesting feature of these changes is that the enzyme response seems to be differential with respect to the substrate used. Ligands with a phosphate group at C-1 only induce the conformation of the enzyme with increased accessibility of the fast-reacting Cys<sup>289</sup> residue, whereas ligands with C-6 phosphate or both C-1 and C-6 phosphates induce the conformation of aldolase with decreased reactivity of this group. The mechanism of the aldolase reaction could not be unequivocally determined (Lai et al., 1974; Periana et al., 1980). One of the interesting features of the aldolase reaction is the pronounced difference in the kinetic properties observed for Fru-1,6-P<sub>2</sub> and Fru-1-P (Penhoet et al., 1967). On the basis of published mechanisms of the reaction, it is difficult to explain this difference (especially the ~10 times higher value of \( V_{\text{max}} \) for Fru-1,6-P<sub>2</sub> in comparison to Fru-1-P) (Buczylik et al., 1980).

Our results offer a simple explanation for this phenomenon.
We propose that the chemical mechanism of fructose-1,6-P₂ and fructose-1-P cleavage is the same and that the enzyme discriminates between these two substrates by adopting a different conformation upon the formation of an enzyme-substrate complex, resulting in different kₐ values for these substrates. In this sense, substrate-induced conformational changes in aldolase are syncretic in nature (Birchmeier and Christen, 1977). The old concept of “induced-fit” (Koshland, 1958) would be, in this case, used by the enzyme to control substrate specificity.

**Long-range Effects Induced by Intrasubunit Cross-linking and C-terminal Tyr** removal — Cross-linking of the aldolase subunit with the Cys²⁷—Cys³ⁱ⁸ pair results in the significant perturbation of enzyme conformation that is propagated to Cys³⁹ and Cys⁴⁰. Therefore, this is a good example of long-range effects in aldolase. This modification also results in the complete loss of enzyme activity and a loss of ability to bind active-site ligands. This is quite remarkable since Cys²⁷ and Cys³⁸ are not in the active site of aldolase (Heyduk and Kochman, 1986; Takahashi et al., 1988; Sygusch et al., 1987) (Fig. 4). It can then be concluded that cross-linking of the enzyme results in a very dramatic rearrangement of the active-site region and that binding of active-site ligands is inhibited. This rearrangement of the α-helix with the flexible peptide. Also, the Cy~Cys~ pair is close to a hinge region connecting the

Therefore, the formation of a Cys²⁷—Cys³¹ bridge is likely to affect the flexibility of this peptide. It seems that freedom of movement of the C-terminal portion of the polypeptide chain in the aldolase molecule is a vital element of enzyme catalysis and is required for the correct transition state structure of the enzyme-substrate complex.

**Acknowledgment** — The critical comments of Prof. James C. Lee are greatly appreciated.

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