Allosteric communication in mammalian muscle aldolase

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Mixed disulphide formation in the presence of oxidized glutathione reversibly inactivates rabbit skeletal muscle aldolase. Inactivation is allosteric, preferentially modifying Cys-72 on the surface of the aldolase homotetramer distant from active-site locations and subunit interfaces. Ion-exchange chromatography fractionates partly inactivated aldolase into three distinct enzymic species: unmodified enzyme, inactive fully modified enzyme corresponding to one thiol reacted per subunit, and inactive singly modified enzyme in which only one thiol has reacted. Acid-precipitable enzymic intermediates formed in the presence

of substrate, D-fructose 1,6-bisphosphate, and product, dihydroxyacetone phosphate, indicates that active site binding is unaffected upon modification. The absence of enamine carbanion formation in the presence of substrate but not product is consistent with mixed disulphide formation's blocking –C–C– cleavage and/or subsequent D-glyceraldehyde 3-phosphate release. Inactivation upon single subunit modification and substrate protection against modification denotes that the blocked step is associated with a long-range conformational transition involving highly co-operative subunit behaviour.

INTRODUCTION

Skeletal muscle aldolase is a ubiquitous and abundant glycolytic enzyme that reversibly cleaves D-fructose 1,6-bisphosphate (Fru-1,6-P₂) into triose phosphates, D-glyceraldehyde 3-phosphate (glyceraldehyde-P) and dihydroxyacetone phosphate. The catalytic mechanism has been examined in some detail for the rabbit isoenzyme and involves Schiff base intermediate formation upon substrate attachment [1]. Subsequent cleavage of the substrate C₃–C₄ bond releases glyceraldehyde-P and leaves an enamine carbanion bound at the active site [2]. Protonation of the carbanion and hydrolysis of the resultant Schiff base releases the obligate product dihydroxyacetone phosphate, regenerating the enzyme. Rabbit muscle aldolase is a homotetramer and has been shown to participate in compartmentalized reaction systems [3–5]. Initial rate kinetics provide no evidence for subunit co-operativity or allosteric effects.

Results from peptide binding assays [6] as well as antibody binding studies [7] have suggested negative subunit co-operativity; inactivation studies with small ligands were consistent with strong subunit interactions in aldolase isoenzymes [8]. Binding of inositol polyphosphates to aldolase was also sigmoidal and analysis of the binding isotherm yielded evidence for slight negative subunit co-operativity [9]. Indication of long-range effects in aldolase has come from intra-subunit cross-linking experiments of aldolase subunits involving disulphide formation between Cys-72 and Cys-338 [10], residues distant from the active site. Disulphide cross-linking inactivated the enzyme by eliminating the binding of active-site ligands and modified the reactivities of other thiols, Cys-239 and Cys-289, also distant from the cross-linking site.

Thiol oxidation by oxidized glutathione in rabbit muscle aldolase forms a limited number of mixed disulphides that inhibit catalytic activity [11]. Steric encumbrances caused by the relatively large molecular dimensions of oxidized glutathione would imply the preferential modification of specific surface thiol residues. Furthermore locations of the modified cysteine residues are distinct from the active-site location given the absence of active-site thiol residues [12]. The effects of surface residues on

catalytic activity would allude to a potential allosteric mechanism of activity modulation in muscle aldolase consistent with protein–protein interactions in compartmentalized reaction systems. Surprisingly, the modification of a surface thiol, Cys-72, of only a single aldolase subunit was sufficient to abolish catalytic activity in all subunits. Mixed disulphide formation did not abrogate active-site binding but inhibited substrate cleavage and/or glyceraldehyde-P release.

MATERIALS AND METHODS

Fru-1,6-P₂ trisodium salt and fructose 1-phosphate hexamethyl ammonium salt were purchased from Sigma Chemical Co. Dihydroxyacetone phosphate and glyceraldehyde-P were bought as the dimethyl ketal form and deprotected in accordance with the supplier's protocol (Sigma Chemical Co.). NADH, NADPH, oxidized glutathione and 5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) were obtained from Boehringer Mannheim Co. Other reagents and chemicals were of at least reagent grade. Fructose 1,6-P₂ aldolase from rabbit skeletal muscle (specific activity 9–10 units/mg) and glutathione reductase from yeast were purchased from Boehringer Mannheim. Recombinant human muscle aldolase, as well as mutants C72A and C338A and the double mutant C72A/C338A [13], were gifts from Dr. Y. Takasaki and Dr. K. Hori (Saga Medical School, Saga, Japan). Aldolase and mutants were purified to homogeneity by affinity elution ion-exchange chromatography [14]. Specific activities of these mutants, 9.8–10.4 units/mg, were similar to those of rabbit and human skeletal muscle aldolases. Aldolase enzymic activity was determined by a coupled assay by following NADH oxidation spectrophotometrically at 340 nm [15]. Protein concentration of aldolase was determined using the bicinchoninic acid protein reagent assay (Pierce Chemical Co.). Phosphate content of enzymic complexes was analysed in triplicate by the procedure of Ames [16] and used typically 1 mg or more of enzyme. Unless stated otherwise all other assays and determinations were performed in duplicate.

Oxidized glutathione incorporation and thiols released in the

Abbreviations used: Fru-1,6-P₂, D-fructose 1,6-bisphosphate; glyceraldehyde-P, D-glyceraldehyde 3-phosphate; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

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presence of glutathione reductase were analysed by Nbs_2 titration of residual thiol groups [17,18]. Enzymic determination of glutathione incorporated by aldolase, approx. 1 mg per reaction, was modified by the addition of a trichloroacetic acid precipitation step (10%, v/v) before assay of the released glutathione. The pellet was discarded and the supernatant was neutralized to pH 7.4 by the addition of 5 M NaOH and then assayed by Nbs_2 titration for thiol released. Thiols were quantified by following the release of the Nbs_2^- chromophore (ϵ_{412} $13.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) at 412 nm for at least 30 min at 25 °C.

Rabbit skeletal muscle aldolase and recombinant human aldolases were inactivated in the presence of various concentrations of oxidized glutathione at room temperature in 100 mM triethanolamine/HCl buffer (pH 7.4)/5 mM EDTA. After incubation for a predetermined period, the reaction was diluted 5–10-fold and assayed for residual activity. Unreacted oxidized glutathione was removed by extensive dialysis of the protein sample against 20 mM Bistris buffer, pH 6.7. At this pH, inactivation was considerably diminished. The ligand concentration range used in the inactivation studies varied between 100 μM and 10 mM ligand.

Inactivated rabbit skeletal muscle aldolase (0.3 mg/ml) was incubated in the presence of 5 units of glutathione reductase, 2 mM NADPH, 100 mM triethanolamine/HCl buffer, pH 7.5, and 5 mM EDTA for 5 min at room temperature. Aliquots were removed and assayed for activity. Inactivated aldolase was also incubated with 10 mM dithiothreitol for 30 min at room temperature before activity assay.

Partly inactivated rabbit skeletal muscle aldolase was fractionated by a combination of anion-exchange and cation-exchange chromatography as described previously [8]. The incubation mixture was diluted with an excess of 20 mM Bistris buffer, pH 6.7, and applied to an anion-exchange (Mono Q 5/5 HR FPLC) column (Pharmacia) previously equilibrated in the same buffer. Unreacted protein was collected in the flow-through fraction. Modified protein was released, on application of a 1 M NaCl linear salt gradient (flow rate 1 ml/min), at 0.45 M NaCl. Released protein was dialysed overnight against 20 mM K^+ /Tes buffer, pH 7.2, and applied to a cation-exchange (Mono S 5/5 HR FPLC) column (Pharmacia) previously equilibrated in the same buffer. Fully modified protein was not adsorbed on the cation exchanger. A 1 M NaCl linear salt gradient, flow rate 1 ml/min, released the partly modified protein at 0.30 M NaCl. The stoichiometry of glutathione modification of muscle aldolase was determined by Nbs_2 titration of residual aldolase thiol groups as well as by thiols released on incubation in the presence of glutathione reductase. The estimated variation in stoichiometry determination was ± 0.1 thiol modified per aldolase tetramer. Molecular masses were determined by gel filtration with a Superose 12 FPLC column (Pharmacia), equilibrated with 100 mM Tris/HCl buffer (pH 7.5)/50 mM NaCl, as the stationary phase.

To investigate protein–ligand complex formation [19,20], the phosphate content of the acid-precipitable N-glycoside enzyme complexes with dihydroxyacetone phosphate and Fru-1,6- P_2 were analysed. Specific formation of the aldolase–dihydroxyacetone phosphate complex was also monitored by absorbance at 240 nm [21] and by the reduction of ferricyanide [22]. The absorbance assay consisted of 500 μM dihydroxyacetone phosphate made up in a 1 ml cuvette (1 cm light path) containing 40 μM aldolase subunits dissolved in 20 mM Tris/HCl buffer, pH 7.4. Oxidation of the dihydroxyacetone enamine carbanion in the presence of 1.43 mM potassium ferricyanide used aldolase (50 $\mu\text{g}/\text{ml}$) dissolved in 20 mM glycine/Tris buffer, pH 7.3, and was determined both under steady-state conditions (1 mM Fru-

1,6- P_2) and under equilibrium conditions (1 mM dihydroxyacetone phosphate).

RESULTS

Inactivation by oxidized glutathione

Incubation of rabbit skeletal muscle aldolase in the presence of oxidized glutathione resulted in the progressive loss of catalytic activity. Activity loss was first-order in time (Figure 1) and demonstrated saturation behaviour consistent with a minimal scheme where the inactivator forms a reversible complex with the enzyme, described by the dissociation constant K_d , and then reacts covalently to modify the enzyme with rate constant k_i [22]. Values of $8.0 \times 10^{-3} \text{ min}^{-1}$ and 294 μM were obtained for k_i and K_d respectively from a double-reciprocal plot (results not shown). Breaks or deviations from linearity in the double-reciprocal plot were not observed over the concentration range tested.

The inactivation of aldolase activity was fully reversible by incubation in the presence of dithiothreitol or glutathione reductase and was consistent with thiol group modification, resulting in mixed disulphide formation. Full protection against inactivation was afforded in the presence of the substrates, Fru-1,6- P_2 (1 mM) and fructose 1-phosphate (10 mM), but not by the products, dihydroxyacetone phosphate (1 mM) or glyceraldehyde-P (1 mM). Full inactivation results in the loss of $4 (\pm 0.1)$ thiol residues per aldolase tetramer at saturating concentrations of oxidized glutathione. On the basis of the reactivity towards Nbs_2 [17], inactivation is consistent with the modification of a slowly reacting thiol group under non-denaturing conditions.

Thiol residue modified

Activity loss for the various recombinant human aldolase mutants incubated in the presence of oxidized glutathione is also shown in Figure 1. No loss of activity is observed for the double mutant of recombinant human aldolase C72A/C338A. Activity

Figure 1 Inactivation of muscle aldolase by oxidized glutathione

Rabbit skeletal muscle aldolase and recombinant human muscle aldolases were incubated for various durations in the presence of 1 mM oxidized glutathione (a) or 5 mM oxidized glutathione (b). Residual activity as a function of initial activity is shown for rabbit muscle (●) and for the various mutant recombinant human muscle aldolases C338A (▲), C72A (■) and C72A/C338A (▼). Inactivation progress curves for human muscle aldolase were as observed for rabbit muscle aldolase (results not shown).

loss for the C338A point mutant is, as for rabbit muscle aldolase, first-order in time and saturable. Under conditions where catalytic activity for the point mutant C338A is undetectable, activity of the point mutant C72A remains readily detectable. Catalytic activity for either mutant is fully restored by incubation in the presence of dithiothreitol. From the crystal structure of rabbit skeletal muscle aldolase [12], the two cysteine residues are located on the aldolase subunit surface with the thiol of Cys-72 being freely accessible to solvent, whereas the Cys-338 thiol has a somewhat restricted solvent accessibility. The thiols of these residues are separated by 9.2 Å and are located approx. 25 Å distant from subunit interfaces and their proper active site, which is located in the subunit β -barrel centre. A comparison of human muscle aldolase and rabbit skeletal muscle aldolase reveals no apparent differences in their tertiary structures at Cys-72 and Cys-338 locations [24]. Furthermore progress curves corresponding to inactivation of recombinant native human muscle aldolase by oxidized glutathione were indistinguishable from those of rabbit muscle aldolase.

Chromatography of inactivated enzymic species

The incorporation of a glutathione moiety by rabbit skeletal muscle aldolase near physiological pH increases the negative charge on the protein. The resultant surface charge incorporation was used as the basis for separation of the modified from the unmodified enzyme with anion-exchange chromatography as described previously [8]. The modified species, which is adsorbed on the stationary phase, is devoid of catalytic activity and is eluted as a single fraction. The stoichiometry of glutathione incorporated by this fraction increased progressively with incubation time from a minimum of one glutathione incorporated to a maximum of four glutathiones incorporated per aldolase tetramer. Chromatography of the modified enzyme population utilized cation-exchange chromatography as described previously [8]. At pH 7.2 the modified tetrameric population was separated by cation-exchange chromatography into two distinct fractions. On the basis of glutathione content, the fraction adsorbed on the stationary phase represents $1 (\pm 0.1)$ glutathione incorporated per aldolase tetramer (designated A_1). The concentration of this fraction increased initially before progressively decreasing as a function of incubation time. The fraction not retained by the cation exchanger increased steadily with time corresponding to fully modified enzyme having $4 (\pm 0.1)$ glutathiones incorporated per aldolase tetramer (designated A_4) (results not shown) and on the basis of the chromatographic separation is consistent with one modification per aldolase subunit. Molecular sieving of both A_1 and A_4 fractions yielded molecular masses for the modified aldolase species consistent with a tetrameric subunit composition. Detection of the singly modified fraction, A_1 , preceded that of the fully modified fraction, A_4 , and was not inconsistent with a precursor relationship where modification of a single subunit is prerequisite for full subunit modification. Partly modified tetramer populations suggesting stoichiometries other than that of singly modified tetramers were not observed within experimental error (results not shown). Neither substrates nor products protected the singly modified aldolase species from subsequent modification to fully modified enzyme. Glutathione exchange with unreacted thiols was unlikely because even on prolonged storage demonstrable activity was not observed in either A_1 or A_4 species.

Equilibrium enzymic complexes

Acid denaturation/precipitation of covalent aldolase–ligand intermediates traps imine and carbinolamine complexes formed

in the presence of Fru-1,6- P_2 and dihydroxyacetone phosphate under equilibrium conditions [19,20]. On the basis of phosphate content, all acid-denatured protein precipitates yielded equilibrium stoichiometries consistent with previously reported values of 0.51 Fru-1,6- P_2 and 0.45 dihydroxyacetone phosphate molecules bound per aldolase subunit [19,20]. No significant differences in phosphate content could be detected in the distribution of protein-bound N-glycosides between native and inactivated A_1 and A_4 enzymic species. The formation of the enamine carbanion complex in the presence of dihydroxyacetone phosphate was also compared between the unmodified and modified enzymic species. An A_{240} of 0.05 is characteristic of enamine carbanion formation [21], and oxidation by ferricyanide of the enamine carbanion yielded rate constants of $3.3 \pm 0.1 \text{ s}^{-1}$ for both species and agreed within experimental error with results previously reported for native rabbit muscle aldolase [22]. In the presence of Fru-1,6- P_2 , oxidation by ferricyanide of the enamine carbanion in the unmodified enzyme yielded rate constants of $3.2 \pm 0.1 \text{ s}^{-1}$, whereas no ferricyanide reduction was observed for both A_1 and A_4 species, suggesting that formation of the enamine enzymic complex occurred after –C–C– bond cleavage was blocked.

DISCUSSION

Rabbit skeletal muscle aldolase is inactivated allosterically in the presence of the biological oxidant oxidized glutathione. Inactivation by oxidized glutathione shows saturation kinetics indicating a preferred oxidized-glutathione-binding locus on each aldolase subunit and consistent with the reversible loss of one slowly reacting thiol residue per aldolase subunit. Mutation of the slowly reacting thiol residues Cys-72 and Cys-338 to alanine residues [13] indicates, on the basis of the inactivation kinetics, Cys-72 as the residue reacting more rapidly with oxidized glutathione in recombinant human muscle aldolase. Preferential modification of the Cys-72 thiol is consistent with its location at the subunit surface and its unhindered accessibility to solvent, in contrast with the Cys-338 thiol, which has less solvent accessibility and whose modification exhibits slower inactivation kinetics. Inactivation implies an allosteric mechanism because Cys-72 is located 25 Å distant from its proper active site location as well as subunit interfaces. The reversible loss of catalytic activity of rabbit muscle aldolase differs from a previous finding in which irreversible secondary modification was observed during progressive modification by oxidized glutathione [11]. Differences in manufacturer's storage protocol for the enzyme have, in our hands, affected aldolase inactivation behaviour and might be the basis for the apparent discrepancy.

Ion-exchange chromatography resolved partly inactivated skeletal muscle aldolase into distinct enzymic species, consistent with the covalent incorporation of a charged ligand on the aldolase subunit surface. Only enzymic species having incorporated one and four glutathione molecules, A_1 and A_4 species, were detected. Had oxidized glutathione modified both Cys-72 and Cys-338 residues, stoichiometries different from one or four subunits reacted would be predicted. Stoichiometries corresponding to the presence of other than singly or fully modified species were not observed within experimental error. Furthermore the modification of both Cys-72 and Cys-338 would require an A_1 species having a subpopulation of tetramers in which only Cys-338 was modified. On the basis of the inactivation kinetics, shown in Figure 1, the modification of Cys-338 does not lead to a significant decrease in activity; hence activity should have been detected in such a mixture, which was not the case. By the same observation, thiol exchange between Cys-72 and Cys-338 can

also be ruled out. The absence of catalytic activity from the A_1 species suggests that aldolase subunits are not kinetically independent.

Cross-linking of Cys-72 and Cys-338 by S–S bridge formation has been shown to abrogate binding at the active site, thereby compromising catalytic activity [10]. The decrease in the interatomic S–S distance from 9.2 to 2 Å owing to S–S cross-linking imposes, minimally, a conformational deformation at the Cys-72 and Cys-338 structural location. Obstruction of active-site binding as a result of this cross-linking requires long-range propagation of the induced conformational deformation to the active-site location, modifying active-site geometry. Oxidized glutathione inactivation, in contrast, does not compromise active-site binding. The enzymic species A_1 and A_4 are fully competent in Schiff base formation with substrate or product and promote enamine carbanion formation in the presence of dihydroxyacetone phosphate, all indistinguishable from the unmodified species. The modified species do, however, differ from the unmodified enzyme in the absence of steady-state enamine carbanion formation in the presence of Fru-1,6-P₂. This difference is consistent with the blockage of substrate C–C cleavage and/or subsequent glyceraldehyde-P release and is sufficient to explain the decrease of catalytic activity in both singly and fully modified enzymic species. The data, however, cannot discriminate which of the two kinetic steps is compromised because the blockage of glyceraldehyde-P release could interfere sterically with oxidation by ferricyanide of the enamine carbanion intermediate. Substrate C–C cleavage and/or glyceraldehyde-P release represent the rate-limiting mechanistic step in rabbit muscle aldolase and have been postulated to reflect a slow conformational transition [20,25].

Protection against glutathione modification of Cys-72 by substrate requires the existence of long-range communication of a conformational change in muscle aldolase that is stabilized by active-site attachment. Previous studies confirm that substrate protects the slowly reacting thiol residues Cys-72 and Cys-338 against chemical modification [10,26,27]. The long-range conformational change is not required for substrate or product active-site binding because Schiff base formation occurs with substrate or product and enamine carbanion formation takes place in the presence of dihydroxyacetone phosphate and cannot be distinguished from the unmodified enzyme. The inability to protect against inactivation by the reaction products glyceraldehyde-P and dihydroxyacetone phosphate makes the conformational change specific to the formation of enzyme–substrate-like intermediates and, because Schiff base formation is not compromised, the conformational change must be associated with substrate cleavage and/or glyceraldehyde-P release. Modification of Cys-72 in the absence of substrate thus interferes with or prevents a conformational change required for catalysis and thereby inactivates the enzyme allosterically. Protection against modification by fructose 1-phosphate, at concentrations below saturation ($K_m \approx 12$ mM), would further require conformational changes to be communicated from occupied to unoccupied subunits and, together with the lack of activity of the A_1 species, reflects highly co-operative subunit behaviour in the aldolase tetramer. Coupling between subunits is significant because substrate does not protect against further modification of A_1 to A_4 . The results thus argue that in rabbit muscle aldolase the rate-limiting kinetic step necessitates a conformational transition that

must be relayed in a highly co-operative fashion between all subunits of the aldolase tetramer. Highly co-operative or concerted subunit behaviour, required for cleavage and/or subsequent product release, would not be detected from initial rate kinetics.

Mixed disulphide formation has established the existence of a functional link in aldolase, relating the active site located at the β -barrel centre of aldolase and a thiol modification at its subunit surface approx. 25 Å distant, that is capable of mediating substrate cleavage and/or product release. Activity modulation as a result of perturbations or interactions with the enzyme surface, distant from the active site, provides a possible conceptual framework for the regulation of aldolase activity through adsorption/desorption with cellular constituents [28]. Tight subunit coupling would be particularly advantageous whenever aldolase activity needs to be regulated through interactions involving a single subunit, as has been postulated, in compartmentalized reaction systems such as skeletal muscle triads [3] and the membrane-localized glycolytic pathway in erythrocytes [4], as well as with F-actin [5,28].

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