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A selective reaction of fructose bisphosphate aldolase with fluorescein isothiocyanate in chicken muscle extracts

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The present work describes the selective covalent modification of fructose bisphosphate aldolase in crude extracts of chicken breast muscle by fluorescein 5′-isothiocyanate (5′-FITC) at pH 7.0 and 35°C. The modification was observed after 1 min while no other major soluble protein was labeled even after 30 min. We calculated that *ca.* one 5′-FITC molecule was incorporated into each aldolase tetramer after a 30 min reaction which resulted in a minimal loss of enzyme activity. The "native" structure of aldolase was required for the selective modification by 5′-FITC since high pH, high temperature, and ionic detergents either inhibited or prevented the reaction of 5′-FITC with aldolase. Certain metabolites (ATP, ADP, CTP, GTP, FBP) and erythrosin B also inhibited the 5′-FITC modification of aldolase. In contrast, F-6-P, AMP, NADH, and NAD^+ as well as free lysine and most importantly, the 6′-isomer of FITC exhibited no competition with 5′-FITC for the labeling of aldolase. Alone, the 6′-isomer of FITC did not exhibit preferential reaction when combined with aldolase. 5′-FITC-labeled and -unlabeled aldolases were not distinguished by their ability to bind to muscle myofibrils (MFs) or by their abilities to refold following reversible denaturation in urea. Structural analysis revealed that 5′-FITC-labeled a tryptic peptide corresponding to residues 112–134 in the primary structure of aldolase, a peptide that does not contain lysine, the amino acid believed to be the primary target of this reagent. Unlike chicken and rabbit muscle aldolases, chicken brain and liver aldolase isoforms along with several other aldolases derived from diverse biological sources did not exhibit this highly selective modification by 5′-FITC.

Keywords: aldolase; FITC; physiological; nucleotide; isoforms; chemical modification; native

INTRODUCTION

Chemical probes have been used in a variety of ways to examine the structure and function of proteins as well as their locations in tissues and cells. Some probes and their uses include: (1) antibodies specifically directed against protein epitopes, (2) lectins which bind proteins containing bound carbohydrate moieties, and (3) reagents (fluorescent, photoreactive, radioactive, etc.) containing functional groups which are known to react with specific amino acids.

"Affinity" labeling of enzymes is generally performed with synthetically prepared substrate analogs which can form covalent bonds with "essential" functional groups, thus facilitating identification of "active site" amino acids. A classic example of this is the inactivation of chymotrypsin with *N*-tosyl-L-phenylalanylchloromethyl ketone (TPCK). TPCK not only blocks the entire active site of chymotrypsin with its positioning group but, upon alkylation of histidine residue 57, it prevents the proton accepting function of this essential residue (Shaw, 1970).

General chemical reagents have also been used to specifically modify functional groups (amines, sulphhydryl, etc.) required for protein function. In this case, the essential functional groups appear to be much more reactive ("hyperreactive") to these "nonspecific" reagents than are other similar functional groups. Therefore, in many cases, general reagents can behave like "affinity" labels. For example, treatment of ribonuclease with iodoacetate, a "general" chemical reagent, destroys the catalytic activity of the enzyme by the specific alkylation of either histidine residue 12 or 119 suggesting that these two residues are required for ribonuclease activity (Crestfield *et al*., 1963). In the case of chymotrypsin, diisopropyl fluorophosphate (DFP), a "general" phosphorylating agent, reacts specifically with serine residue 195 and renders the enzyme completely inactive. Chymotrypsin and several other proteins were subsequently grouped into a class termed the serine proteases due to their related inactivation by DFP at a "hyperreactive" serine residue (Creighton, 1984).

Fluorescein isothiocyanate (FITC), a "general" chemical reagent, has been termed the "pre-eminent" reagent for the fluorescent labeling of proteins (Haugland, 1992). Fluorescein-conjugated proteins have been used in studying cell morphology (Yoshimura *et al*., 1963; Lopatin and Voss, 1971; Butcher and Weissman, 1980), distribution/migration of cells (Haar *et al*., 1987), hormones (Vilar *et al*., 1964), and cellular components (Underfried, 1962),

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Abbreviations used: *BMCS, breast muscle crude supernate; FBP, fructose bisphosphate; FITC, fluorescein isothiocyanate.*

intra- and inter-molecular distances between proteins (Miki, 1987), fluorescein-ligand-mediated specific protein interactions (Sukhodolets *et al*., 1989), and the dynamics of protein conformational changes (Peerce and Wright, 1984). Although FITC is generally considered to preferentially react with non-protonated, primary amines (Nairn, 1976), there are reports that isothiocyanates can react with other functional groups including alcohols, thiols, and carboxylic acids (The Chemistry of Functional Groups: The Chemistry of Cyanates and Their Thio Derivatives, 1977). Isothiocyanates are also known to react with phenols (Haugland, 1992).

The properties of a protein may be altered by FITC modification, either directly by modification of "essential" residues or indirectly by causing conformational alterations in the "native" protein. For example, anti-streptolysin O antibody (Wood *et al*., 1965), insulin (Halikis and Arquilla, 1961), fibrinogen (Pappenhagen *et al*., 1962), Ca^{2+}-ATPase (Pick and Karlish, 1980), cytochrome P-450_{scc} (Tuls *et al*., 1989), and phosphorylase kinase (Sotiroudis and Nikolaropoulos, 1984) have all been shown to lose protein function following FITC conjugation. In the case of the ATPase, inactivation of enzyme activity was caused by modification of a highly reactive lysine, presumably located at the ATP-binding site of the enzyme (Farley *et al*., 1984; Kirley *et al*., 1985). Also, a highly selective modification of lysine 61 of G-actin eliminated the ability of G-actin to self-polymerize into F-actin (Burtnick, 1984). However, the addition of phalloidin allowed FITC-labeled G-actin to recover its ability to self polymerize, showing that lysine 61 was not directly involved in actin–actin subunit interactions (Miki, 1987). In some cases, (i.e., ribonuclease, cytochrome oxidase, plant catalase, lysozyme (Drobnica, 1967), and β-amylase (Kojima and Naroba, 1971)), modification of proteins with isothiocyanate probes had little effect on their functional properties.

The present studies were initiated by a chance observation which showed that, under mild *in vitro* conditions (pH 7.0, 35°C), the 5′-isomer of FITC (5′-FITC) specifically modifies the abundant glycolytic enzyme fructose bisphosphate (FBP) aldolase in homogenates (12 000*g* soluble proteins) of chicken breast muscle. This was a surprising observation since all major muscle proteins contain similar weight percentages of many amino acids, including lysine, and most of them function in the same metabolic pathway (i.e., glycolysis).

In the present work, we: (1) describe mild reaction conditions which result in the specific modification of aldolase by 5′-FITC, (2) show that this specific modification requires aldolase to be in its "native" conformation, (3) show that a number of specific metabolites inhibit the modification of aldolase by 5′-FITC, (4) compare a number of functional properties of "native" and modified aldolase, (5) demonstrate that 5′-FITC modified a tryptic peptide (corresponding to residues 112–134 in the primary structure of rabbit muscle aldolase) which is devoid of a lysine residue, and (6) show that subtle differences in the structures of aldolases from different biological sources have profound effects on their abilities to be specifically modified by FITC.

EXPERIMENTAL PROCEDURES

Materials

Materials used in this research included: P11 phosphocellulose (Whatman Ltd., Florham Park, NJ), Coomassie protein assay reagent used in Bradford analyses (Pierce, Rockford, IL), TPCK treated trypsin (Worthington Biochemical Corp., Freehold, NJ), and fluorescein 5′-isothiocyante (5′-FITC), fluorescein 6′-isothiocyante (6′-FITC), and rabbit muscle FBP aldolase (Sigma, St. Louis, MO). The following dyes were obtained from Invitrogen Corp., (Carlsbad, CA): 7-amino-4-methylcoumarin-3-acetic acid, succinimidyl ester (AMCA), 5′-(and-6′)-carboxyfluorescein, succinimidyl ester (CFSE), 5′-iodoacetamidofluorescein (IAF), and tetramethylrhodamine 5′ (and 6′)-isothiocyanate (TRITC). Any biochemicals not listed above were purchased from Sigma. Other chemicals used were of reagent grade.

Organisms

All tissues used were obtained from either male or female freshly decapitated chickens of at least 3 weeks in age. Breeds used were white leghorns from McIntyre's Poultry (Lakeside, CA) and either New Hampshire reds or a black Cornish "cross" from Cebe Farms (Ramona, CA).

Preparation of muscle extracts

Breast muscle from freshly sacrificed chickens was weighed and immediately placed into 20 volumes of 50 mM KH_2PO_4/K_2HPO_4, 1 mM EDTA, pH 7.0. The tissue was then homogenized with a high speed Sorvall Omni-mixer (DuPont Instruments) using two 30 s high speed bursts with a 15 s break in between the bursts. Homogenates were centrifuged at 12 000*g* for 15 min at 5–10°C. The supernates (designated breast muscle crude supernate or BMCS) were extensively dialyzed at 4°C against 10 mM Tris-HCl, 1 mM EDTA, pH 7.0, stored at 4°C, and used within 2 weeks of preparation. No significant loss of aldolase catalytic activity or change in the reaction of aldolase with 5′-FITC was noticed during storage of extracts for up to 2 months after preparation.

Labeling of proteins with fluorescent probes

The reaction of BMCS or purified proteins with fluorescent probes was performed as follows: (1) solutions of 50 μM FITC, IAF, AMCA, CFSE, and TRITC were prepared in 10 mM Tris-HCl, pH 7.0, (2) an equal volume of probe solution was mixed with the protein sample and incubated at 35°C for 30 min unless otherwise indicated, and (3) the reactions were stopped by addition of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis sample buffer (0.24 mM Tris-HCl, pH 6.8, 7.8% SDS, 19.5% 2-mercaptoethanol or 2-Me, and 0.01% bromophenol blue) and boiling for 2 min. For investigating the effect of pH on 5′-FITC labeling, 60 μM solutions of 5′-FITC were prepared in 10 mM bis-Tris propane-HCl buffer at pH 6.5, 7.0, 7.5, 8.0, or 9.0. BMCS was equilibrated by dialysis against these same buffers prior to reaction with the corresponding 5′-FITC solutions as described above. All probe solutions were stored in the dark at 4°C until used.

Electrophoretic procedures

Samples were analyzed in 10% polyacrylamide slab gels containing 0.1% SDS using the reagents suggested by Laemmli (1970). After electrophoresis at a constant current of 65 mA, gels were immediately UV-illuminated and photographed. Then, gels were fixed in 10% acetic acid:45% methanol and stained for protein with 0.2% (w/v) Coomassie blue R250. After destaining, gels again were photographed. Electrophoresis in nondenaturing

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5% basic polyacrylamide slab gels was performed as described by Gabriel (1971) at a constant current of 35 mA. The basic gels were further treated as described above.

Phosphocellulose column chromatography

Phosphocellulose (PC) column chromatography was used to separate BMCS into specific enzyme fractions and to isolate aldolase A_4 essentially as described by Petell *et al*. (1981). Approximately 10 ml of BMCS was applied to a PC column ($1.5 \times 15\ cm^2$) and unbound protein (termed the "breakthrough fraction") was eluted with 10 mM Tris-HCl, 1 mM EDTA, pH 7.3. Protein was detected by measuring absorbance at 280 nm. The column was then washed with 0.1 M Tris-HCl, 1 mM EDTA, pH 7.8 and the protein peak eluted (termed the "Tris" fraction) was collected. Aldolase was specifically eluted by the addition of 1 mM FBP in the 0.1 M Tris-HCl buffer and proteins still bound to the PC column were eluted with 1 M NaCl contained in the 0.1 M Tris-HCl buffer ("salt" fraction). The above four fractions were then extensively dialyzed at 4°C against 10 mM Tris-HCl, pH 7.0. Labeled aldolase A_4 was isolated from BMCS, previously reacted with 5′-FITC at 35°C for 30 min, by affinity elution from PC as described above.

Isolation of other aldolases

Rattlesnake (*Crotalus oreganos helleri*), frog (*Rana pipiens*), bass (*Morone saxatilis*, Walbaum), shark (*Squalus acanthias*), and lobster (*Homarus americanus*) tail muscle aldolases as well as chicken (*Gallus gallus*) liver aldolase were isolated as previously described by Swain and Lebherz (1986). Chicken (*G. gallus*) brain (Lebherz, 1975), wheat (*Triticum* spp.) germ (Heil and Lebherz, 1978), and spinach (*Spinacia oleracea*) leaf chloroplast (Lebherz *et al*., 1984) aldolases were isolated as previously described.

Assays

Aldolase catalytic activity was determined using the coupled enzyme assay of Lebherz and Rutter (1975). Activity was expressed as μmoles FBP cleaved/min at 25°C. Concentrations of pure unlabeled aldolase samples were estimated spectrophotometrically using an extinction coefficient of $0.91_{A_{280}}$ per mg protein/ml (Baranowski and Niederland, 1949).

The method suggested by Tuls *et al*. (1989) was used to estimate fluorescein content of 5′-FITC-labeled aldolase. Since FITC absorbs at 280 nm (Wilderspin and Green, 1983), the protein content of 5′-FITC-labeled aldolase was determined by the Coomassie protein assay (Bradford) suggested by the Pierce Co. using a standard curve generated with purified chicken muscle aldolase.

Myofibril-binding studies

Chicken breast muscle myofibrils (MFs) depleted of bound glycolytic enzymes were prepared as described by Hong (1987). Briefly, the procedure involved homogenization of muscle in 10 mM Tris-HCl, 1 mM 2-Me, pH 7.0 containing either 50 mM phosphate or 150 mM KCl. Homogenates were filtered, MFs were collected by centrifugation, and the MF pellets were washed by resuspension in buffer containing 1% Triton X-100 to facilitate removal of membrane lipids (Solaro *et al*., 1971). After further washing to remove Triton, MFs were resuspended in 20 volumes of 10 mM Tris-HCl, 1 mM 2-Me, pH 7.0 and were stored at 4°C until used.

The ability of 5′-FITC-labeled aldolase to bind MFs was investigated using the procedure described by Hong (1987). About 140 μg of unlabeled or 5′-FITC-labeled aldolase was mixed with 250 μl aliquots of MFs in microcentrifuge tubes (final volume 350 μl). The samples were incubated at room temperature for 10 min and MFs containing bound aldolase were collected by centrifugation (14 500*g*, 2 min). (Unbound aldolase appeared in the supernate.) After washing, MF pellets were resuspended in 350 μl of 50 mM phosphate, 1 mM 2-Me, 1 mM EDTA, pH 7.0 to release the bound aldolase which appeared in the supernate after centrifugation.

The 5′-FITC labeling of aldolase bound to depleted MFs was performed as follows: (1) chicken muscle aldolase (140 μg) was incubated with MFs (washed and resuspended in 10 mM Tris-HCl, pH 7.0) in microcentrifuge tubes (final volume 300 μl) as described above, (2) the mixture was centrifuged (14 500*g* for 2 min) and the supernate, containing unbound aldolase, was discarded, (3) the MF pellet, which contained bound aldolase, was again washed, resuspended in 200 μl 10 mM Tris-HCl (pH 7.0), and 100 μl of 50 μM 5′-FITC (in 10 mM Tris, pH 7.0) was added and (4) after incubation at 35°C for 30 min, bound 5′-FITC-labeled aldolase was released from the MFs as described above.

Formation of chicken muscle and brain aldolase "hybrid" tetramers

The ability of 5′-FITC-labeled chicken muscle aldolase A subunits to form "hybrid" tetramers with unlabeled chicken brain aldolase C subunits was investigated following the procedure described by (Kent and Lebherz, 1984). Unlabeled or 5′-FITC-labeled aldolase A_4 was dissociated to subunits in 5.8 M urea whereas aldolase C_4 was dissociated in citric acid at pH 2.3. Recovery of aldolase activity upon reassociation of subunits into tetramers was between 40 and 50%. "Hybrids" were analyzed by charge separation in a nondenaturing 5% basic polyacrylamide gel using the procedure described above.

5′-FITC-binding site on chicken muscle aldolase

Unlabeled and 5′-FITC-labeled aldolases were carboxymethylated according to the method of Angeletti *et al*. (1971) using a 100-fold molar excess of iodoacetamide, instead of iodoacetic acid, presuming eight cysteine residues per aldolase subunit (Tolan *et al*., 1984). The S-carboxymethyl aldolases were extensively dialyzed against 0.1 M NH_4HCO_3 (pH 9.3) and were then digested with trypsin using the method of Lebherz *et al*. (1984). At the end of the second incubation period (8 h instead of 15 h), the samples were evaporated to dryness in a Speed-Vac Concentrator (Savant Instruments).

The aldolase tryptic peptides were resolublized in 0.1% (v/v) trifluoroacetic acid (TFA) and were subjected to reverse phase HPLC on a Zorbax C_{18} column (4.6 mm × 25 cm; DuPont) using a linear gradient from solvent A (0.1% TFA) to 5% solvent A and 95% solvent B (0.1% TFA in 80% acetonitrile) over 65 min at a flow rate of 1 ml/min. Peptide elution was monitored at 225 nm and any fluorescence was visualized by UV-illumination after alkalinizing aliquots of the fractions to pH ~9.5 with NH_3. The fluorescent fraction was diluted with 0.1% TFA to ~10%

acetonitrile and was further resolved on the Zorbax C_{18} column as above with the exception of allowing the linear gradient between solvents A and B to occur for over 130 min instead of 65 min. Peptide elution and fluorescence were detected as above.

Ion-exchange HPLC was performed as described by Shields and Glembotski (1988) with the following alterations. The fluorescent peptide fraction obtained from the second reverse phase HPLC was resolved on a Bio-Sil TSK CM-2-SW column (4.6 mm × 25 cm; Rainin) developed with a linear ammonium formate (pH 5.0) gradient in acetonitrile over 65 min at a flow rate of 1 ml/min. Peptide elution and fluorescence were detected as above. The fluorescent fraction was evaporated to dryness in a Speed-Vac Concentrator. Finally, the chromatographically purified 5′-FITC-tryptic peptide was subjected to amino acid sequence analysis using a Porton Instruments liquid phase model 2020 protein sequencer (Beckman Instruments). Fractions of the eluent were collected and any fluorescence was detected as above.

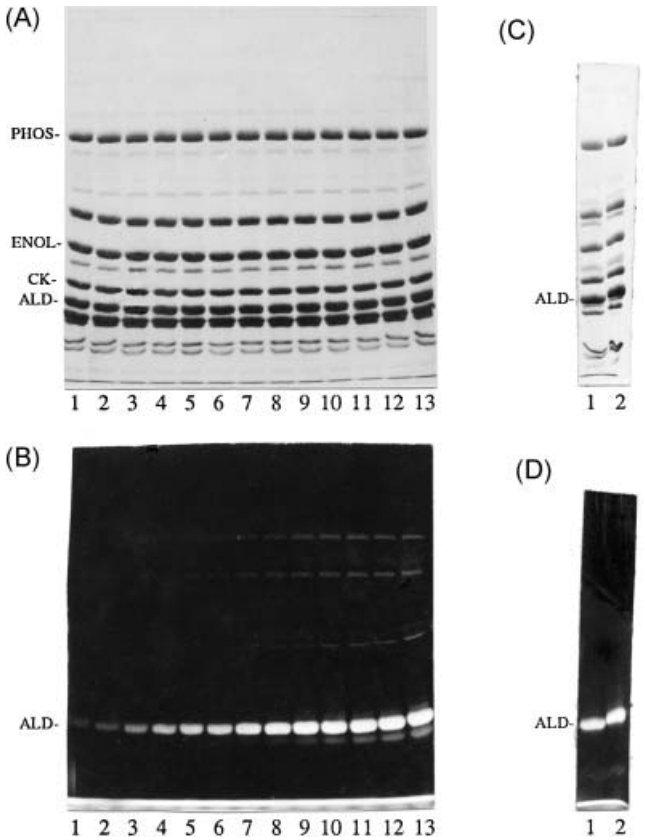

Figure 1. Reaction of BMCS proteins with 5′-FITC at 35°C versus time. (A) shows a Coomassie blue stained SDS polyacrylamide gel which contained 30 μl aliquots of BMCS reacted with an equal volume of 5′-FITC (50 μM in 10 mM Tris-HCl, pH 7.0) for 0.5, 1, 2, 3, 4, 5, 10, 15, 20, 30, 45, 60, and 75 min (lanes 1–13, respectively). (B) is the same gel UV-illuminated before Coomassie blue staining. (C) shows a Coomassie blue stained gel which contained 40 μl aliquots of BMCS in the absence (lane 1) or presence (lane 2) of 50 mM lysine reacted with 5′-FITC for 10 min at 35°C. (D) is the same gel in part C UV-illuminated before Coomassie blue staining. Abbreviations are: PHOS-phosphorylase (92 kD), ENOL-enolase (50 kD), CK-creatine-P kinase (43 kD), and ALD-aldolase (40 kD) (Petell *et al.*, 1981).

RESULTS

Preferential labeling of aldolase in muscle extracts with 5′-FITC

The selective modification of aldolase in BMCS with 5′-FITC at 35°C, pH 7.0, is illustrated in Figure 1. As shown, aldolase was the only major protein labeled under these conditions (Figure 1A,B) and apparently maximal labeling of aldolase occurred within 15 min. Consequently, a 30 min reaction time was chosen for most of the 5′-FITC labeling experiments performed below.

The primary amine functional group of lysine has a pK_R of ~10 (Creighton, 1984) so, at pH 7, only about 1 out of 1000 lysine R groups would be expected to be in the reactive state (non-protonated) and as shown in Figure 1D, the presence of 50 mM free lysine did not prevent the specific modification of aldolase by 5′-FITC. Thus, the rapid and specific labeling of aldolase under the mild conditions used here is apparently due to the reaction of 5′-FITC with a "hyperreactive" side chain(s) of the aldolase molecule.

Specific labeling of aldolase by 5′-FITC is not due to a unique position of aldolase in a multi-protein complex

One possible explanation for the hyperreactivity of 5′-FITC toward aldolase in BMCS would be that aldolase occupies a unique position in a multi-protein complex. Indeed, a number of workers have postulated the existence of a glycolytic enzyme complex in muscle extracts (Clarke and Masters, 1973; Melnick and Hultin, 1973; Ovadi, 1988; Pagliaro and Taylor, 1988). Consequently, if aldolase was located on the exterior of such a multi-protein complex, perhaps it could "shield" the labeling of proteins located inside. This possibility was ruled out in the following experiment. BMCS proteins were resolved into four different fractions as described under Methods. As shown in Figure 2A,B, the major BMCS proteins in fractions which did not contain aldolase (lanes 1, 2, and 4) were not labeled with 5′-FITC, while, as expected the purified aldolase (lane 3) was. Furthermore, after reconstituting BMCS by mixing all of the separated

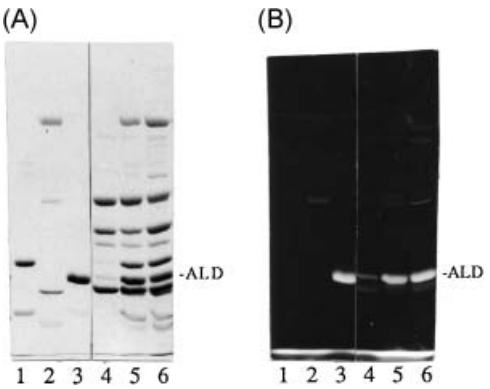

Figure 2. 5′-FITC labeling of separate BMCS protein fractions. Using phosphocellulose chromatography, major BMCS proteins were separated into specific fractions and labeled with 5′-FITC. (A) shows a Coomassie blue stained gel which contained 30 μl of "breakthrough," 30 μl of "Tris," 12 μl of aldolase, and 20 μl of "salt" peaks reacted with 5′-FITC (lanes 1–4, respectively). Lanes 5 and 6 contained 78 μl of the recombined fractions and 4.7× diluted BMCS, respectively. (B) is the same gel UV-illuminated before Coomassie staining.

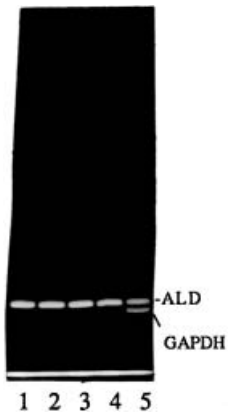

Figure 3. Effect of varying pH on 5′-FITC-labeling of BMCS proteins. BMCS equilibrated at varying pHs with 10 mM bis-Tris propane-HCl was reacted with 5′-FITC at 35°C. The displayed UV-illuminated gel contained 30 μl aliquots of reaction mixtures incubated at pH 6.5, 7.0, 7.5, 8.0, and 9.0 (lanes 1–5, respectively). GAPDH, glyceraldehyde-3-P dehydrogenase (37.3 kD) (Petell *et al.*, 1981).

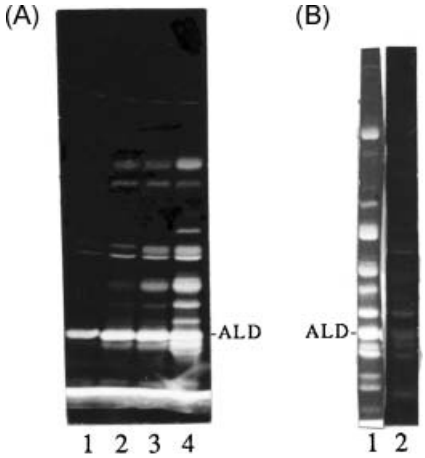

Figure 4. Reaction of BMCS proteins with 5′-FITC at different temperatures. (A) shows a UV-illuminated gel which contained 30 μl aliquots of BMCS reacted with 5′-FITC for 30 min at 25, 35, 45, and 55°C (lanes 1–4, respectively). (B) shows a UV-illuminated gel which contained 30 μl aliquots of BMCS reacted with 5′-FITC at 100°C for 1 min (lane 1) and reacted at 100°C for 5 min prior to reaction with 5′-FITC for 30 min at 35°C (lane 2).

fractions back together, aldolase again was found to be the only protein specifically labeled by 5′-FITC (lane 5).

Specific labeling of aldolase appears to require the "native" structure

The specific labeling of aldolase by 5′-FITC occurred only under conditions which preserved enzyme catalytic activity. Aldolase displays a pH optima for catalytic activity between pH 6.5 to around pH 8.5 (Penhoet *et al.*, 1969) and Figure 3 shows that the specific labeling of aldolase in BMCS by 5′-FITC also occurs over this pH range. Although an increase in the labeling of proteins would be expected at higher pH values (due to the presence of a higher population of non-protonated ε-NH_2 groups on lysine side chains), the intensity of aldolase labeling at pH 9 was actually decreased. It is also interesting to note that, at pH 9, considerable labeling of glyceraldehyde-3-P dehydrogenase occurred.

The time courses of heat inactivation of aldolase catalytic activity and inactivation of specific labeling of aldolase by 5′-FITC were also directly correlated (data not shown).

Also, aldolase catalytic activity and ability to be specifically labeled by 5′-FITC were both retained in the presence of the nonionic detergent Triton X-100 (1%) but both were lost in the presence of the cationic detergent *n*-dodecyltrimethyl-ammonium bromide (1%) and the anionic detergent SDS (1%; data not shown).

A different mechanism of 5′-FITC labeling occurs at elevated temperatures

The selective labeling of aldolase in BMCS does not occur at high temperature. That is, as reaction temperatures are increased from 25°C to 55°C, additional proteins in BMCS become labeled by 5′-FITC (Figure 4A). At reaction temperatures higher than 70°C, essentially all proteins become highly labeled within 5 min. For example, note the 5′-FITC protein labeling pattern of BMCS treated with 5′-FITC at 100°C for 5 min (Figure 4B, lane 1). However, after heat denaturation (100°C, 5 min) no major BMCS protein, including aldolase, could subsequently be labeled by 5′-FITC at 35°C (Figure 4B, lane 2). Thus, it appears that, at high temperature, 5′-FITC can modify all proteins in BMCS but the mechanism by which it does so is distinct from that responsible for the selective labeling of aldolase at physiological temperatures.

Other fluorescent probes do not specifically modify aldolase in BMCS

To determine if aldolase is simply more susceptible to modification by chemical reagents, fluorescent probes other than 5′-FITC were tested for their abilities to selectively modify BMCS proteins.

Since all major BMCS proteins contain cysteine residues (Protein Identification Resource, National Biomedical Research Foundation, Washington, DC), they all possess the potential to be modified by the sulfhydryl reactive probe, IAF. However, as shown in Figure 5A, creatine kinase and glyceraldehyde-3-P dehydrogenase became highly labeled with IAF after 15 min while aldolase was not significantly labeled by this reagent even after 90 min. Additional fluorescent probes which are believed to react with primary amines (i.e., AMCA, CFSE, and TRITC) did not "selectively" label aldolase in BMCS (data shown only for CFSE in Figure 5B).

Most importantly, 6′-FITC did not rapidly or selectively label aldolase in BMCS (Figure 5C, lane 1) but, as expected, 6′-FITC did extensively label all major BMCS proteins when the reaction temperature was raised to 100°C (lane 2). Finally, no difference in the fluorescence intensity of BMCS aldolase reacted with 5′-FITC in the presence or absence of a threefold molar excess of 6′-FITC occurred (data not shown), suggesting that 6′-FITC does not have a high affinity for the 5′-FITC-binding site on aldolase. The results presented in this section demonstrate that the precise electronic ring structure of 5′-FITC and position of its isothiocyanate group are required to selectively label "native" aldolase in BMCS.

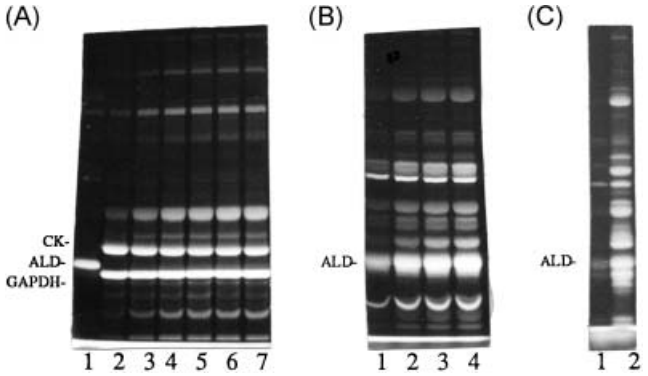

Figure 5. Reaction of BMCS proteins with IAF, CFSE, and 6′-FITC. (A) shows a UV-illuminated gel which contained 30 μl aliquots of BMCS reacted with 5′-FITC for 30 min (lane 1) and IAF for 1, 5, 15, 30, 60, and 90 min (lanes 2–7, respectively) at 35°C. (B) shows a UV-illuminated gel which contained 50 μl aliquots of BMCS reacted with CFSE for 1, 5, 15, and 30 min (lanes 1–4, respectively) at 35°C. (C) shows a UV-illuminated gel which contained 20 μl aliquots of BMCS reacted with 6′-FITC for 30 min at 35°C (lane 1) and 2 min at 100°C (lane 2).

Inhibition of the specific 5′-FITC modification of aldolase by certain metabolites

The above results demonstrate that 5′-FITC has a remarkable ability to specifically label aldolase in BMCS under very mild conditions. We therefore considered the possibility that this fluorescent probe may be mimicking a natural biomolecule in breast muscle and we tested the ability of certain metabolites to inhibit this selective modification. At the onset, it should be emphasized that, unlike metabolites which react non-covalently and transiently with aldolase, 5′-FITC forms a covalent bond. Consequently, 5′-FITC will eventually "win" in competition experiments as the time of incubation is increased. For example, as shown in Figure 6A, although 2 mM ATP inhibited 5′-FITC labeling of aldolase when incubations were performed up to 60 min (lanes 7–11), inhibition was not observed when the incubation time was raised to 4 h (lane 12).

As shown in Figure 6B, ATP (lanes 2–4) and, to a lesser extent, ADP (lanes 5–7) successfully inhibited the labeling of aldolase by 5′-FITC during a 2 min reaction time while AMP (lanes 8–10) had no effect, even at concentrations as high as 250 μM. Although CTP (50 μM) and GTP (50 μM) were also found to be effective inhibitors, the dinucleotides NAD^+ and NADH at concentrations as high as 1000 μM had no effect on the intensity of labeling of aldolase by 5′-FITC during a 2 min reaction (data not shown). Furthermore, 2′, 4′, 5′, 7′-tetraiodofluorescein (TIF or erythrosin B) binds with high affinity to the adenine nucleotide-binding sites (four per tetramer) on rabbit muscle aldolase (Neslund *et al.*, 1984). We found that 50 μM TIF also inhibited 5′-FITC labeling of aldolase in BMCS to a similar extent as did 25 μM ATP (data not shown). Finally, the aldolase substrate FBP, but not the non-substrate F-6-P, inhibited the labeling of BMCS aldolase by 5′-FITC although, unlike ATP, high concentrations (>125 μM) of FBP were required to cause similar inhibition of labeling (data not shown).

Comparisons of selected properties of unlabeled and 5′-FITC-labeled aldolase

5′-FITC-labeled aldolase was isolated from BMCS previously incubated with 5′-FITC (concentration was determined to be in excess, data not shown) for 30 min at 35°C by affinity elution from PC. The labeled aldolase was revealed to be about 10% ($9.8 \pm 5.5\%$) less active than the unlabeled enzyme and contained 1.1 ± 0.63 5′-FITC molecules per aldolase tetramer.

No differences between labeled and unlabeled aldolases were detected on the basis of the following: (1) abilities to bind to muscle MFs; also unlabeled aldolase bound to MFs could be labeled with 5′-FITC (data not shown) and (2) abilities to form heterotetramers by combination with unlabeled chicken brain aldolase C subunits following reversible dissociation of the tetrameric species (Figure 7).

Location of the 5′-FITC-binding site on chicken muscle aldolase

Protein sequencing was performed in an attempt to locate the amino acid residue(s) in aldolase labeled by 5′-FITC. Freshly

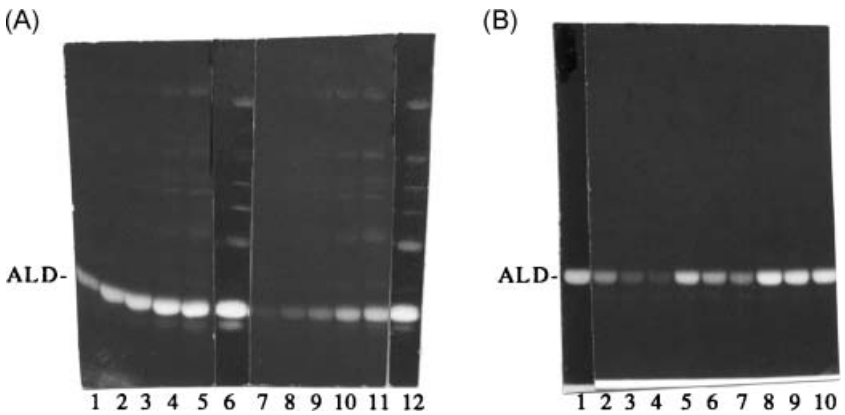

Figure 6. Reaction of BMCS proteins with 5′-FITC in the presence of ATP, ADP, and AMP. (A) shows a UV-illuminated gel which contained 20 μl aliquots of BMCS containing either 0 (lanes 1–6) or 2 mM ATP (lanes 7–12) reacted with 5′-FITC for 2, 5, 10, 30, 60, and 240 min (left to right, respectively) at 35°C. (B) shows a UV-illuminated gel which contained 21 μl aliquots of BMCS (lane 1) and BMCS containing 50, 250, or 500 μM ATP (lanes 2–4, respectively), ADP (lanes 5–7, respectively), or AMP (lanes 8–10, respectively) reacted with 5′-FITC for 2 min at 35°C.

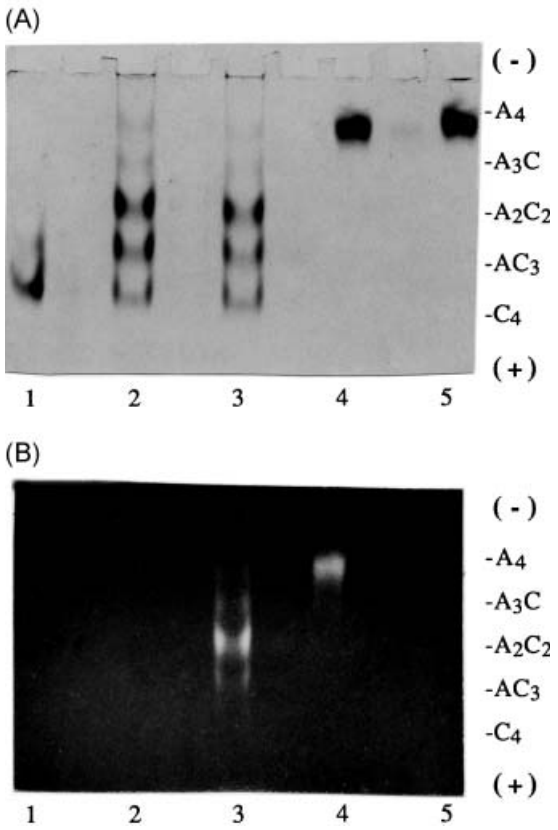

Figure 7. "Hybridization" of 5′-FITC-labeled chicken aldolase A_4 with unlabeled C_4. (A) shows a Coomassie blue stained nondenaturing 5% basic polyacrylamide gel which contained ~14 μg of aldolase C_4 (lane 1), ~30 μg of unlabeled or 5′-FITC-labeled aldolase A_4 "hybridized" with aldolase C_4 (lanes 2 and 3, respectively), and ~18 μg of 5′-FITC-labeled (lane 4) or unlabeled (lane 5) aldolase A_4. (B) is the same gel UV-illuminated before Coomassie staining.

isolated unlabeled and 5′-FITC-labeled aldolases were carboxymethylated and these derivatized enzymes were then exhaustively digested with trypsin. The resulting tryptic peptides were resolved by reverse phase HPLC. As shown by the peptide elution profiles depicted in Figure 8A,B, the only difference in these profiles was the appearance of an additional peak in the HPLC chromatogram of the 5′-FITC-labeled aldolase "tryptics." This additional peak, which represents a combination of peptide bond and 5′-FITC absorbance, can be clearly observed in the expanded sections of the HPLC chromatograms shown in Figure 8C,D. This additional peak contained essentially all of the fluorescence as judged by visual examination of the fractions (after alkalinization) under a UV light. The fluorescent fraction was purified further on a second reverse phase column. This preparation contained two peptides, as determined by amino acid sequence analysis, and the two peptides were separated on an ion-exchange HPLC column. The fluorescent-peptide peak which appeared in the column "breakthrough" fraction was subjected to further sequence analysis which showed that this peptide was more than 95% pure and corresponded to amino acid residues 112–134 of the rabbit muscle aldolase subunit (refer to Figure 10). Note that this peptide does not contain a lysine residue so we conclude that the site of modification by 5′-FITC is not a primary amino group.

During sequence analysis, fluorescence was observed in the fractions corresponding to the "wash" solution following cycle 9, and within both the HPLC resolution and "wash" fractions of cycles 10 and 11, while a relatively small amount of fluorescence was also detected in cycle 12. These cycles displayed amino acid peaks which corresponded to Asp, Gly, Glu, and Thr, respectively (data not shown).

Reaction of other aldolases with 5′-FITC

Finally, we investigated whether different isoforms of aldolase could also be selectively labeled with 5′-FITC. Unlike muscle aldolase A_4, neither chicken liver (B_4) nor brain (C_4) aldolases were labeled by 5′-FITC to any considerable extent at 35°C and pH 7.0 (Figure 9B). However, as expected, all three chicken aldolase isoforms were labeled with 5′-FITC when the reaction temperature was raised to 100°C (Figure 9C).

Although rattlesnake and shark muscle (A_4) aldolases could be labeled with 5′-FITC at 35°C, they were not as intensely labeled as the rabbit or chicken muscle aldolases. Further, A_4 aldolases from other animal (frog, bass, and lobster) or plant (wheat germ and spinach chloroplast) origins were not labeled by 5′-FITC. Finally, as expected, all of the selected aldolases were extensively labeled by 5′-FITC when the reaction temperature was raised to 100°C (data not shown).

DISCUSSION

Results of the present studies show that 5′-FITC rapidly and selectively labels aldolase in BMCS at pH 7.0 and 35°C. Even though primary amines are believed to be the predominant "targets" for modification by 5′-FITC, excess amounts of free lysine did not inhibit the selective labeling of aldolase. This observation was most likely due to the reaction pH being considerably below the recommended minimal pH of ~9.0 for the reaction between FITC and free lysine (Maeda *et al*., 1969). Furthermore, the "native" structure of aldolase appears to be required for this selective labeling, as judged by the corresponding effects pH, temperature, and ionic detergents had on aldolase catalytic activity and on its ability to be specifically labeled by 5′-FITC. On the other hand, all major BMCS proteins could be labeled by 5′-FITC at 100°C suggesting that a second, non-specific, labeling mechanism predominates at higher temperatures.

We ruled out the possibility that selective labeling of aldolase in BMCS by 5′-FITC could have been due to aldolase being located at the exterior of a putative multi-protein complex in BMCS thus acting to "shield" other BMCS proteins from being modified by 5′-FITC. That is, after removal of aldolase from BMCS, the remaining major proteins still were not labeled under these mild conditions.

In contrast to 5′-FITC, several other amine reactive probes (AMCA, TRITC, and CFSE), as well as the sulfhydryl reactive probe, IAF, did not specifically modify aldolase in BMCS at pH 7.0 and 35°C. The extreme sensitivity of aldolase to be labeled by 5′-FITC was further vividly demonstrated by our observation that 6′-FITC failed to label aldolase under these mild conditions, even though, like 5′-FITC, it could label all BMCS proteins by the non-specific mechanism at high temperature. Thus, it is apparent that the specific labeling of aldolase in BMCS requires a precise interaction

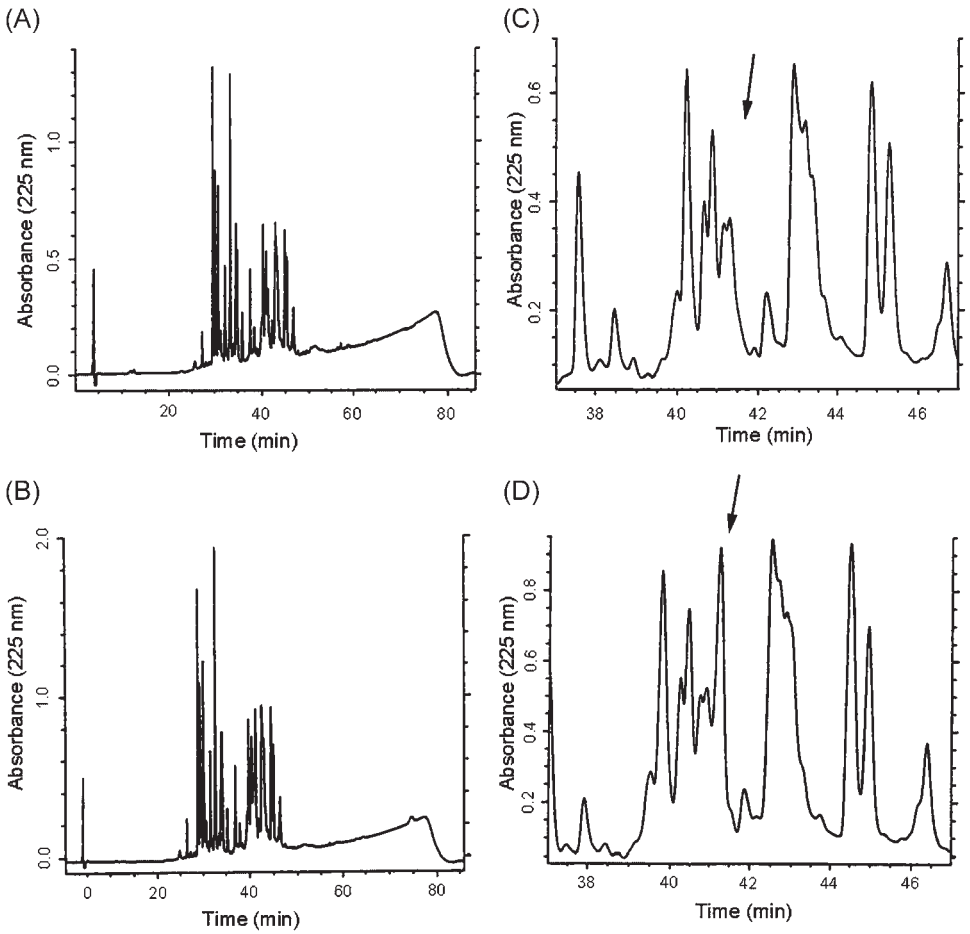

Figure 8. Reverse phase HPLC resolution of unlabeled (A) and 5′-FITC-labeled (B) aldolase tryptic peptides. (C) and (D), respectively, show expansions (37–47 min) of the HPLC chromatograms depicted in (A) and (B). (The arrow indicates the position of the peak which corresponds to the putative 5′-FITC-labeled peptide.)

between a "hyperreactive" labeling site(s) on native aldolase molecules and 5′-FITC.

Results of competition experiments showed that ATP, CTP, GTP, and TIF, a putative nucleotide analog, were particularly potent inhibitors of the specific labeling of aldolase by 5′-FITC, while ADP and the aldolase substrate FBP were less effective inhibitors. AMP, the dinucleotides NAD^+ and NADH, F-6-P (a non-aldolase substrate), and 6′-FITC were not observed to inhibit the reaction of 5′-FITC with aldolase in BMCS. Since 5′-FITC has been suggested to be a nucleotide analog, these results indicate that aldolase may contain a mononucleotide pocket that is specific for at least diphosphorylated nucleotides and either proximal to or allosterically related to the active site of the enzyme. (The results suggest that fluorescein may be employed for the affinity purification of aldolase.) Although other major BMCS proteins contain nucleotide-binding domains, the inability of 5′-FITC to specifically label these proteins may reflect the absence of a precise interaction between an amino acid "R" group on these proteins and 5′-FITC (a situation analogous to the failure to selectively label aldolase with 6′-FITC).

The selective incorporation of approximately one 5′-FITC molecule per aldolase tetramer (after a 30 min reaction at 35°C, pH 7.0) had no appreciable effect on a number of functional properties of the enzyme, including its specific catalytic activity (a loss of $9.8 \pm 5.5\%$), interaction with MFs, and ability to refold and establish precise subunit/subunit contacts after reversible denaturation in urea. Taken together, these results suggest that the specific modification of chicken muscle aldolase by 5′-FITC does not have a pronounced effect on the gross 3D conformation of this enzyme. Since excess FITC was present after the 30 min

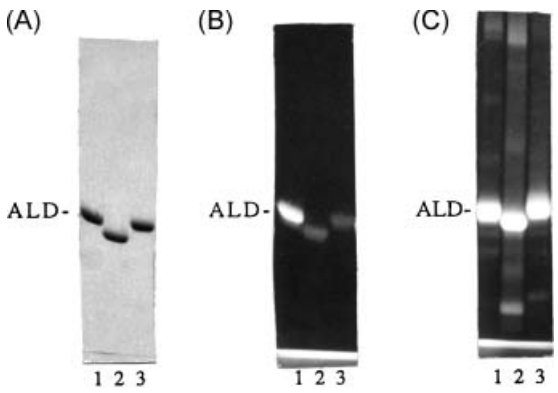

Figure 9. Reaction of chicken aldolase isoforms with 5′-FITC. (A) shows a Coomassie blue stained gel which contained ~20 μg each of A_4, B_4, and C_4 aldolases reacted with 5′-FITC (lanes 1–3, respectively) at 35°C. (B) is the same gel in part A UV-illuminated before Coomassie blue staining. (C) shows a UV-illuminated gel which contained the three aldolase isoforms treated with 5′-FITC at 100°C for 5 min.

Aldolase:	Partial amino acid sequence:
Gallus gallus (Chicken) A	GVVPLAGTDG**E**TTTQGLDGLMER
Oryctolagus cuniculus (Rabbit) A	**112-** GVVPLAGTNGETTTQGLDGLSER **-134**
Chicken B	GTAPLAGTNGETTIQGLDKLAER
Chicken C	GVVPLAGTDGETTTQGLDGLSER
Xenopus tropicalis (Western Clawed frog) A	GVVPLAGTNGETTTQGLDGLSER
Xenopus laevis (African Clawed frog) A	GVVPLAGTNGETTTQGLDGLSER
Danio rerio (Zebrafish) A	GVVPLAGTNGETTTQGLDGLYER
Callorhinchus callorynchus (Elephantfish) A	GVVPLAGTNGETTTQGLDGLSER
Cephaloscyllium umbratile (Blotchy swell shark) A	GVVPLAGTNGETTTQGLDGLNER
Spinacia oleracea (Spinach) cytosol	GTVELAGTNGETTTQGLDGLAQR
Spinach chloroplast [precursor]	GWLPLPGSNDESWCQGLDGLACR

Figure 10. Comparison of amino acid sequences of the 5′-FITC-labeled tryptic peptide of chicken aldolase A_4 with corresponding sequences of aldolases from other biological sources. All sequences were retrieved from the Protein sequence database (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD).

reaction with BMCS (data not shown), it can be concluded that the modification involved the binding of approximately one FITC molecule per aldolase tetramer and not the labeling of each subunit in one quarter of the tetramers. (Unlike native or FITC-labeled aldolase A_4 that were both demonstrated to bind MFs, it is of interest to note that the more basic aldolase B_4 and the more acidic aldolase C_4 were neither labeled by FITC at low (35°C) temperature as demonstrated in this study nor do they readily bind to MFs as previously demonstrated in Hong (1987)).

Aldolase is not considered to be an allosteric enzyme since its catalytic activity has been demonstrated to follow Michaelis–Menten kinetics (Lebherz and Rutter, 1973). Thus, it can be implied (and indeed has been demonstrated (Penhoet and Rutter, 1971)) that the tetramer's subunits function "independently" of each other and could therefore be assumed to react with 5′-FITC "independently." But, since it appears that only ∼1 5′-FITC molecule binds per aldolase tetramer, perhaps all four subunits are somehow involved with 5′-FITC recognition and subsequent binding. In addition, the ∼9.8% drop in activity does not correspond to the theoretically predicted 25% drop for the inactivation of a single aldolase subunit within the tetramer.

The 3D structure of rabbit aldolase A_4 at 2.7 Å has been determined by Sygusch *et al.* (1987) and was later expanded to 2 Å resolution by Gamblin *et al.* (1991). The site of 5′-FITC modification of chicken muscle aldolase has been identified as residing in a single tryptic peptide which corresponds to residues 112–134 in the primary structure of rabbit muscle aldolase. Since chicken and rabbit aldolase A subunits presumably have similar primary and tertiary structures, the 5′-FITC-reactive region would correspond to the "loop" located between the "c" β-sheet and "C" α-helix in the 3D structure of these proteins. This would place the 5′-FITC-reactive region of aldolase outside of the active site which is consistent with our observation that the 5′-FITC modification does not appreciably hinder the catalytic activity of the enzyme.

Aldolase A_4 contains one tight and one weak ATP-binding site per aldolase subunit (Kasprzak and Kochman, 1980). Affinity labeling studies using ATP analogs have identified Lys-107 and Tyr-363, implicated to be associated with the active site, as well as Thr-265 to participate in nucleotide binding to aldolase A_4. Nucleotide binding was concomitant with a linear inactivation of rabbit aldolase A_4 and suggested Lys-107 and Tyr-363 to be involved with tight ATP binding. Interestingly, under reaction conditions (pH 8.5) which minimized interaction with the weak nucleotide-binding site, an incorporation of ∼1.8 ATP analogs per aldolase tetramer was observed. Furthermore, reaction with an affinity label was shown to prevent aldolase from binding to PC implicating the nucleotide-binding site to be associated with the active site of the enzyme (Palczewski *et al.*, 1985; Palczewski and Kochman, 1987). (Aldolase binds to PC at its active site (Felicioli *et al.*, 1975).) The sites of modification by the affinity labels are within reasonable proximity to the apparent 5′-FITC reactive peptide on aldolase. But, 5′-FITC-labeled aldolase retains almost complete activity and ability to bind PC. It therefore appears that 5′-FITC (bound to aldolase) does not overlap the active site of aldolase as was suggested for ATP (bound to the tight nucleotide-binding site on aldolase) by Palczewski *et al.* (1985) and Kasprzak and Kochman (1980). It was not clear whether ATP inhibition of labeling of aldolase by 5′-FITC, as observed in this report, was due to direct competition at a common binding site or was due to an allosteric phenomenon.

Within the rabbit aldolase A_4 tetramer, one of the two pairs of subunit contact sites were determined by Sygusch *et al.* (1987) to be located between the symmetry-related "E" and "F" α-helices and the other between the relatively distant "loop" regions which contain mostly hydrophobic residues and, coincidentally, the 5′-FITC-reactive area. Even though the 5′-FITC modification occurs in or near a subunit–subunit contact region of aldolase polypeptide chains, the fluorescein moiety did not hinder formation of hybrid tetramers containing 5′-FITC-labeled A and unlabeled C aldolase subunits.

Of all the aldolases tested, only those from chicken and rabbit muscle were extensively modified by 5′-FITC within 30 min of reaction at 35°C and pH 7.0. Since neither the more basic liver nor more acidic brain isoform (Penhoet *et al.*, 1966) was selectively labeled by 5′-FITC under these mild conditions, it can be concluded that the overall charge on muscle aldolase is not responsible for its higher susceptibility to modification by 5′-FITC.

The primary sequences for several of the evolutionarily diverse aldolases studied here are presently unavailable. However, Figure 10 shows the primary sequence of the tryptic peptide, derived from chicken aldolase A, that was the site of 5′-FITC modification as compared with those of some biologically diverse aldolases. Of particular interest is the fact that the chicken peptide contains no "internal" primary amine "R" groups which precludes a lysine target site for 5′-FITC. This chicken peptide also lacks other potentially FITC-reactive residues including Cys

(Podhradský *et al*., 1979; Haugland, 1992) and Tyr (Haugland, 1992). All of these aldolases have similar amino acid sequences in this region and all contain the conserved, potentially FITC-reactive (The Chemistry of Functional Groups: The Chemistry of Cyanates and Their Thio Derivatives, 1977) amino acid residue Glu-122 (*bold-faced* for chicken aldolase sequence in Figure 10). Asp-120 (perhaps misassigned) of the chicken aldolase is not a likely site for FITC modification since it corresponds to an Asn, which contains a poorly reactive amide group, in the also 5′-FITC-reactive rabbit aldolase. The Thr residues located at positions 119 and 123–125 are also, although unlikely due to relatively low reactivity (Haugland, 1992), candidates for FITC modification. Thus, it is likely that subtle differences in conformations of these highly conserved proteins are responsible for a lack of shared 5′-FITC recognition by aldolases derived from different sources.

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