& Pry, 1980). The parallel decrease (from 96 to 2.69 s^{-1}, 97%) in the NADPH dissociation rate (Table III) provides unequivocal evidence for NADPH release as the rate-limiting step in oxidative decarboxylation, and that inhibition of this step is responsible for substrate inhibition. In a previous study (Reynolds et al., 1978), the slow onset of substrate inhibition was attributed to a slow conformational change of the quaternary malate complex from an active to a less active (7%) form. The first-order time course observed for the transition process (Figure 7) is consistent with such a mechanism which can be written as

$$E_4\text{–}Mn_2^{2+}\text{–}NADPH_2\ (NADP^+)_2\text{–tetramalate} \xrightarrow[t_{1/2} = 0.1\ s]{k = 7\ s^{-1}} E_4'\text{–}Mn_2^{2+}\text{–}NADPH_2\ (NADP^+)_2\text{–tetramalate}$$

in terms of enzyme–ligand complexes defined by the postulated model (Hsu & Pry, 1980). E and E′ are active and partially active enzyme complexes, respectively. Conversely, our results are at variance with the alternative possibility of slow addition of malate in the second-order association reaction which requires a linear dependence of the first-order rate constant for transition (k) on the ligand concentration.

Registry No. NADPH, 53-57-6; Mn, 7439-96-5; L-malic acid, 97-67-6; malic enzyme, 9028-47-1.

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Purification and Properties of Two Hemagglutinins of the Mushroom *Laccaria amethystina*†

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ABSTRACT: Two lectins of different specificity were isolated from the mushroom *Laccaria amethystina* by affinity chromatography with stromas of group O human erythrocytes included in polyacrylamide gel. The more abundant lectin (LAL) was eluted by lactose, which specifically inhibited the agglutination of phenotype A and O erythrocytes and the protozoan *Crithidia fasciculata*. Its molecular weight determined by sodium dodecyl sulfate gel electrophoresis was 17 500, and its structure is apparently monomeric. It appeared homogeneous by electrophoresis but showed microheterogeneity by isoelectric focusing. The other lectin (LAF) was eluted by L-fucose. It possesses anti-H properties, since its agglutinating activity was greater toward phenotype O and A_2 erythrocytes than toward A_1 erythrocytes. Agglutination of erythrocytes and various protozoa was specifically inhibited by L-fucose. Its molecular weight was 16 000, and its structure is apparently monomeric. Its homogeneity was confirmed by polyacrylamide gel electrophoresis and isoelectric focusing.

The occurrence in mushrooms of substances active toward red blood cells of human or animal origin has been recognized since the beginning of the century [for a general review, see Coulet et al. (1970b)]. There are early reports (Ford, 1911; Friedberger & Brossa, 1912; Galli-Valerio & Bornand, 1916) of attempts to relate the toxicity of some species to their hemolytic or hemagglutinant potency. Hemagglutinins occur extensively in mycetes, including edible species; their rate of occurrence in mycetes exceeds that found in higher plants. Systematic explorations covering more than 600 species (Coulet & Merland, 1960a,b; Coulet et al., 1964, 1970a; Merland & Coulet, 1961) have revealed the presence of lectins in about 30% of the higher fungi studied. In addition, specificity with regard to human red blood cells has been found in 18% of the active species: anti A + B (Elo et al., 1951; Guillot & Coulet, 1974), anti B (Coulet & Marche, 1962; Mäkelä et al., 1959), anti B + H (Elo & Estola, 1952), and anti H (Kogure, 1973).

The presence of lectins in mushrooms of the genus *Laccaria* was first demonstrated in *Laccaria amethystina* by Elo &

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Estola (1952) and confirmed by Coulet & Merland (1960a). Other investigations carried out by Raszeja (1958) have revealed anti-H activity in extracts of *Laccaria laccata* of the *proxima* variety.

In a study of the surface structures of various protozoa (Gueugnot, 1980), we were led to use hemagglutinant proteins from *L. amethystina*. We established that the sugars able to inhibit agglutination of these cells belonged to two separate groups [I and II according to Mäkelä (1957)]. We subsequently undertook a systematic study of the inhibition of hemagglutination by a range of sugars covering all four Mäkelä groups. Of these, three proved extremely active, namely, L-fucose (group I), lactose, and *N*-acetyl-D-galactosamine (group II). This finding suggested the presence of two lectins of different specificity in the hemagglutinant extract.

We report here the separate isolation of these two lectins and results of their characterization by electrophoresis and specific inhibition.

Materials and Methods

Affinity Chromatography. Group O human red blood cell stromas included in polyacrylamide gel according to a technique described previously (Bêtail et al., 1975) were placed in a 45 × 2.6 cm column. The use of this type of immunoadsorbant has the advantage of retaining the whole range of proteins able to bind to the structures brought to the surface of the cell membrane. Moreover, since the stromas are simply trapped between the interstices of the gel, there is no likelihood of any change in the glycoproteinic chains.

Crude extract of *L. amethystina* was prepared from mushrooms harvested by us and kept deep-frozen at −18 °C. The carpophores were mashed in phosphate buffer, pH 7.2 (PBS), in the ratio 1:3 (ww), and the extract was collected by centrifugation.

Pure samples of each lectin were obtained by two successive chromatographic separations. First, the column was loaded with 100 mL of crude extract. After a washing with 100 mL of PBS, 200 mL of a solution of 0.05 M lactose or L-fucose was run through the column at a flow rate of 80 mL/h. The eluate was collected in 10-mL fractions, concentrated by dialysis against poly(ethylene glycol) solution, and dialyzed against PBS. The two lectins could be obtained either from two different columns eluted with a single sugar or from a single column successively eluted with the two sugars. We found the first method preferable, as the second requires an additional washing of the column, which lowers the yield of the lectin eluted second. If this second lectin is eluted with fucose, it becomes difficult to detect given its small quantity. In order to eliminate residual pigments and proteins contaminating the lectins thus obtained, the latter were rechromatographed separately under the same conditions as above. Three eluates of each lectin were combined, concentrated down to 100 mL, and loaded onto the column. The eluates from the second chromatography were dialyzed and concentrated, giving purified lectins. Their protein content was determined by the method of Lowry et al. (1951).

Hemagglutination. Hemagglutination trials were conducted by the method of Rosenfield & Haber (1965) on a Technicon autoanalyzer. We used a suspension of human red blood cells from a pool of at least 10 donations at a concentration such that a transmission of about 18% was obtained.

Inhibition of Hemagglutination by Sugars. The sugars chosen among the four Mäkelä groups were used in 0.1 M solution in PBS: (group I) D-arabinose, L-fucose, D-ribose, and D-ribose 5-phosphate; (group II) L-arabinose, D-fucose, D-galactose, D-galactosamine, *N*-acetyl-D-galactosamine (D-GalNAc), α-lactose, β-lactose, melibiose, stachyose, and raffinose; (group III) D-cellobiose, D-fructose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, 2-deoxy-D-glucose, D-lyxose, maltose, D-mannose, sucrose, D-salicin, trehalose, and D-xylose; (group IV) L-glucose, L-rhamnose, L-sorbose, streptomycin, and L-xylose.

The different solutions of sugars were placed in the sample rack of the Technicon autoanalyzer and the agglutinating fractions introduced continuously at a concentration that would cause agglutination of 50% of the group O red cells present. The sugar was mixed with the agglutinin about 10 s before contact with the red cell suspension. The concentration of sugars in the reaction medium was 0.05 M and 0.033 M after the addition of lectin and of red blood cells.

Agglutination and Inhibition of Agglutination of Protozoa. The following strains were used: *Crithidia fasciculata, Culex* ATCC 12857 strain; *Crithidia oncopelti*, strain obtained from ITMA; *Crithidia deanei*, Lump 969 strain provided by Dr. Kimber (Winches Farm Field Station, Saint Albans, England); *Blastocrithidia culicis*, ATCC 30257 strain (adiplosomic F. G. Wallace 1975 strain isolated from *Aedes vexans*); *B. culicis*, ATCC 30258 strain (including an endosymbiot, isolated from *Culex pipiens*); *Leishmania enrietti*, strain obtained from ITMA.

Strains were maintained on brain–heart Difco medium supplemented with 3% defibrinated rabbit blood. The temperature was kept at 20 °C for strains of *Crithidia* and *Blastocrithidia* and at 28 °C for the *Leishmania* strain. The protozoa were collected at their exponential growth stage, i.e., after 48 h in culture for *Crithidia* and *Blastocrithidia* and 96 h for *Leishmania*. The medium was spun at 3700*g* for 10 min. The pellet was then washed twice by resuspension in PBS, pH 7.2. A cell count was performed on a Malassez cell so as to adjust the concentration of protozoa to 1.5×10^4 cells/mm^3.

Lectin solution (0.1 mL) and PBS or 5% sugar solution (0.1 mL) were mixed in the cups of a microtitration plate. After 30 min of contact, 0.1 mL of the cell suspension was added. After a further contact time of 30 min at ambient temperature, a drop of the suspension was placed between slides and examined microscopically. Agglutination was estimated and scored from 0 to ++++ according to the proportion of cells affected.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in the LKB 2117 Multiphor apparatus with the 2103 LKB constant-power supply. Thin-layer gels (250 mm × 115 mm × 2 mm) were polymerized in a glass polymerization chamber (LKB system). Purified lectins were used after adjustment to a concentration of about 1.5 mg/mL.

(*a*) *Electrophoresis.* A pH 7.5 standard gel and Tris [2-amino-2-(hydroxymethyl)-1,3-propanediol]–glycine buffer, pH 8.9, were used. Samples (5 μL of lectins) were placed in preformed troughs by means of an automatic pipet, Pipetman Gilson. Electrophoresis was carried out for 3 h at 250 V. The proteins were stained with Coomassie brilliant blue R-250.

(*b*) *Isoelectric Focusing.* Electrophoresis was performed on 5% polyacrylamide gel according to Karlsson et al. (1973). The gel contained ampholites (LKB 1809 ampholine-carrier ampholites, 40%, w/v) selected to establish a pH gradient from 3.5 to 9.5 as follows: 2.8 mL of ampholine pH 3.5–10, 0.4 mL of ampholine pH 9–11, and 0.2 mL of ampholine pH 4–6 and 5–7. The anode electrolyte was 1 M H_3PO_4 and the cathode electrolyte 1 M NaOH. Samples (10 μL) were applied to 5 mm × 10 mm pieces of Whatman 3MM, which were placed on the gel near the anode. The power supply was set at 1200 V and 35 W maximum for 80 min. The gel was then

Table I: Percentage of Different Red Blood Cells Agglutinated by Lectins Isolated from *L. amethystina*

group of human erythrocytes	agglutinating activity	
	LAL lectin	LAF lectin
O	53	50
A_1	65	18
A_2	75	45

Table II: Inhibition by the Three Most Active Sugars of the 50% Agglutination of Group O Human Red Cells Produced by the Two Lectins Isolated from *L. amethystina*

sugar		inhibiting activity (%)	
		LAL lectin	LAF lectin
lactose:	0.1 M	100	
	0.05 M	85	
	0.025 M	38	
D-GalNAc:	0.1 M	100	
	0.05 M	94	
	0.025 M	62	
L-fucose:	0.01 M		96
	0.005 M		72
	0.0025 M		8

fixed and stained for 15 min at 60 °C with Coomassie brilliant blue R-250 (Karlsson et al., 1973).

(*c*) *Sodium Dodecyl Sulfate (SDS) Electrophoresis.* Electrophoresis was performed in a 7.5% polyacrylamide gel that contained 0.1% SDS. The electrode buffer was 0.1 M phosphate, pH 7.1, with 0.1% SDS.

The proteins were incubated in 1% SDS with or without 1% 2-mercaptoethanol at 100 °C for 10 min. A total of 10 μL of each protein was placed in preformed troughs in the gel. Molecular weight markers for SDS gel calibration were phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactalbumin (14 400) (Pharmacia low molecular weight electrophoresis calibration kit). Electrophoresis was performed at 150 V for 5 h, and the proteins were stained with Coomassie brilliant blue R-250.

Molecular Weight Determination. Gel filtration was performed by using a Sephadex G-150 column (2.6 × 45 cm) equilibrated with PBS. The following served as standards: albumin (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000), and ribonuclease A (13 700).

Results

Isolation of Lectins. Chromatography of 100 mL of crude extract gave, by elution with lactose or L-fucose, agglutinating proteins in about 20 fractions with a peak optical density of 0.10 (reading at 280 nm). After the second chromatography, the lectin eluted by lactose (LAL) was collected in 15 fractions, the optical density of which ranged from 0.05 to 0.12. These fractions, combined and dialyzed against PBS, gave 150 mL of lectin solution with optical density of 0.07. The yield in agglutinating activity toward group O human red blood cells was 40% of that of the crude extract.

The lectin eluted by L-fucose (LAF) in the second chromatography was obtained in 150 mL of a solution with an optical density of 0.03 (the highest optical density observed during fraction collection did not exceed 0.05). The yield in agglutinating activity was about 14% of that of the crude extract. Both lectins were concentrated prior to subsequent tests.

Hemagglutinating Activity. The hemagglutinating potency of a particular solution was expressed as the percentage of red cells agglutinated under the conditions given. Hemagglutinating activity was measured for both lectins toward phenotypes O, A_1 and A_2. We used lectin solutions at 0.10 mg/mL, which corresponds for both lectins to a dose agglutinating 50% of O group cells taken as reference. The results are given in Table I.

LAL lectin was more active toward group A cells, and particularly subgroup A_2, than toward group O cells. LAF lectin showed marked anti-H activity; groups O and A_2 cells were preferentially agglutinated. However, this lectin was able to agglutinate Bombay phenotype cells, Oh Le (a^+b^-), although the reaction was weaker than that with group O cells.

Inhibition of Hemagglutination. LAL lectin was strongly inhibited by lactose (α and β) and by *N*-acetyl-D-galactosamine. On the other hand, the other mono- or disaccharides belonging to the same Mäkelä group and the other sugars were completely inert. LAF lectin was totally and specifically inhibited by L-fucose. Only stachyose and raffinose showed some slight inhibiting activity among the other sugars tested.

The results of tests with progressively decreasing concentrations of active sugars are given in Table II. Evidently, *N*-acetyl-D-galactosamine is markedly more active than lactose in inhibiting LAL lectin; α- and β-lactose were equally active. L-Fucose is clearly markedly inhibiting toward LAF lectin, since even at a concentration of 0.0025 M, it was still active under the conditions of the experiment.

Agglutination and Inhibition of Agglutination of Protozoa. Results of agglutination and its inhibition by lactose and L-fucose are given in Table III. Only *C. fasciculata* was agglutinated by LAL lectin, unlike the other protozoa, which were sensitive to LAF. Lactose specifically inhibited agglutination of *C. fasciculata*, and L-fucose did the same for the other *Crithidia*, *Blastocrithidia*, and *Leishmania* samples, both in the presence of the crude extract and the separate lectins. This indicates the presence of a single category of membrane receptor for all these protozoa, a lactose type for *C. fasciculata* and an L-fucose type for the others.

Purity and Molecular Weight. Figure 1 shows that two quite distinct fractions were separated from the crude extract. Each of these two fractions showed a single electrophoresis band, and they possess opposite charges at pH 8.9. The positive charge of LAF lectin was also evidenced by isoelectric focusing, where it appeared as a single band in the pH zone near 9.5, whereas the LAL lectin showed four bands migrating

Table III: Agglutination of Different Strains of Protozoa by Crude Extract of *L. amethystina* and Isolated Lectins LAL and LAF in the Presence and Absence of Lactose and L-Fucose

protozoa	crude extract			LAL lectin			LAF lectin		
	PBS	lactose	fucose	PBS	lactose	fucose	PBS	lactose	fucose
C. fasciculata	++++	0	++++	++++	0	++++	0	0	0
C. oncopelti	++++	++++	0	0	0	0	++++	++++	0
C. deanei	++++	++++	0	0	0	0	++++	++++	0
B. culicis 30257	++++	++++	0	0	0	0	++++	++++	0
B. culicis 30258	++++	++++	0	0	0	0	++++	++++	0
L. enrietti	++++	++++	0	0	0	0	++++	++++	0

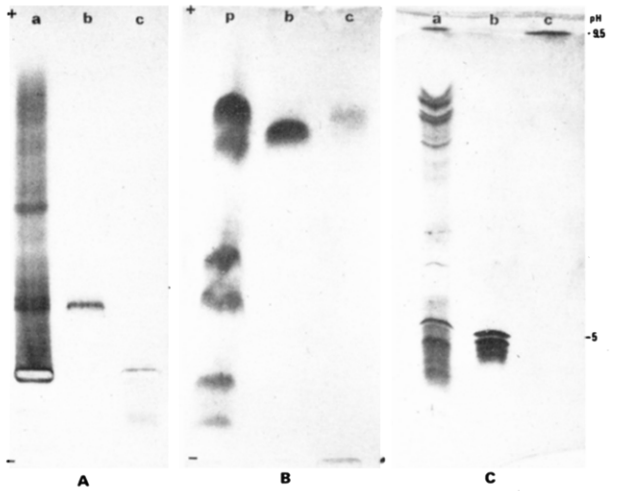

FIGURE 1: Thin-layer polyacrylamide gel electrophoresis of crude extract of *L. amethystina* (a), lectin LAL (b), and lectin LAF (c): electrophoresis in 7.5% gel (A); SDS electrophoresis in 7.5% gel (B) (p is calibration kit proteins); isoelectric focusing in 5% gel with a pH gradient from 3.5 to 9.5 (C). Amount of applied protein was 7.5 μg for electrophoresis and 15 μg for isoelectric focusing and SDS electrophoresis.

in the pH zone near 5. Both lectins are homogeneous by SDS electrophoresis, with an apparent molecular weight of 17 500 for LAL lectin and 16 000 for LAF lectin. These values were unaltered after treatment with 2-mercaptoethanol. The gel-filtration technique gave slightly higher figures than the SDS electrophoresis technique: 19 000 for LAL lectin and 18 000 for LAF lectin.

Discussion

Two lectins were isolated from *Laccaria amethystina* by affinity chromatography with stromas of group O human red blood cells as immunoadsorbant. The more abundant of the two lectins (LAL) was specifically eluted by lactose, which proved a powerful inhibitor of hemagglutination by LAL. The inertness of the other mono- and disaccharides belonging to the same Mäkelä group, except for *N*-acetyl-D-galactosamine, suggests that the site recognized on the glucid chains at the surface of the red cells is fairly large and of precise structure. Such marked dissociation between the activity of lactose and that of the D-galactose-type monosaccharide had also been noted for lectins from other mushrooms, e.g., *Polyporus sulfureus* (Fargeix et al., 1980).

Polyacrylamide gel electrophoresis of the purified lectin showed a single band; however, isoelectric focusing revealed microheterogeneity, since at least four bands appeared. Molecular weight calculated from electrophoretic mobility was about 17 500. The lectin is evidently monomeric, and disulfide bonding is not involved since treatment with 2-mercaptoethanol did not lead to any dissociation into subunits.

The other lectin (LAF) showed an isoelectric point at around 9.5. Its molecular weight was 16 000, and it is apparently composed of a single unit. This lectin showed an anti-H specificity evidenced by its specific elution and inhibition by L-fucose and by its greater activity toward red cells of phenotype O and A_2.

Other fungus extracts also show anti-H properties, e.g., *Xylaria polymorpha* (Tetry et al., 1954), *Clathrus cancellatus* (Silicani et al., 1962), and *L. laccata proxima* (Raszeja, 1958). More recently, Kogure (1975) found in *Pleurotus ostreatus* and *Pleurotus spodoleucus* hemagglutinins with an anti-H specificity similar to that found in *Cytisus sessilifolius* and *Laburnum alpinum*.

Using a dialyzed glycopeptide from submaxillar porcine mucin coupled with Sepharose 4B, Kochibe & Furukawa (1980) have isolated from *Aleuria aurantia* a fucose-binding lectin. Like LAF lectin, *Aleuria* lectin is able to agglutinate Bombay phenotype red cells, which lack the H-determinant structure. Kochibe and Furukawa have shown to the same effect that cells of O Le (a^-b^-) or O Le (a^+b^-) phenotype treated with α(1→2)-fucosidase of *Bacillus fulminans* were still agglutinated, although to a lesser degree, after a 19-h incubation with the enzyme. In contrast, anti-H reagents such as eel serum or *Ulex* lectin do not react with Oh cells or cells treated with α(1→2)-fucosidase. This suggests that LAF and *Aleuria* lectin combine not only with the H-determinant structure but also with other fucoses, these being linked in a different fashion from 1→2 in the case of *Aleuria* lectin.

The anti-H (O) action of a lectin can in fact be the result of different types of linkage with membrane carbohydrates. Lectins of type *Ulex* I or *Lotus*, inhibited by L-fucose and especially strongly by methyl α-L-fucopyranoside, are specific to 1→2 linkage at a nonreducing terminal of the carbohydrate chain and have restricted specificity for the fucose linked to the type 2 chain (Periera et al., 1978). The *P. spodoleucus*, *P. ostreatus* (Kogure, 1975), *C. sessilifolius*, *L. alpinum*, and *Ulex europaeus* II (Voak & Lodge, 1971) lectins are not inhibited by L-fucose but by other sugars or glycosides including lactose. However, when O cells were treated with α(1→2)-fucosidase, they lost agglutinability against the lectins. Kogure puts forward the hypothesis that the L-fucosyl residue at C_2 of D-galactose gives rise to a conformational change of this residue so as to make the β-D-galactosyl linkage more accessible to the lectin. *Aleuria* and *Laccaria* fucose-binding lectins belong to a third category of lectins inhibited specifically by L-fucose and able to link with fucosyl residues other than those determining the H structure. A general review of fucose-binding lectins and their anti-blood group H (O) action has been given by Goldstein & Hayes (1978).

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Registry No. Lactose, 63-42-3; L-fucose, 2438-80-4; *N*-acetyl-D-galactosamine, 1811-31-0.

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Resonance Energy Transfer between the Active Sites of Myocardial-Type Creatine Kinase (Isozyme MB)†

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ABSTRACT: The single reactive sulfhydryl group, located in the active site of each subunit of dimeric creatine kinase from rabbit muscle (isozyme MM), was selectively labeled with 3-(4-maleimidylphenyl)-7-(diethylamino)-4-methylcoumarin (CPM). Isozyme BB, purified to homogeneity from rabbit brain, was conjugated with the sulfhydryl-specific reagent 5′-(iodoacetamido)fluorescein (5′-IAF). Spectral analyses demonstrated that 1.8 mol of CPM and 1.9 mol of 5′-IAF had reacted per mol of protein. Labeled isozymes were combined, denatured in 8 M urea, and renatured by dialysis, producing the parent labeled homodimers and forming the heterolabeled hybrid dimer, creatine kinase MB. Similar hybridizations were performed to prepare singly labeled hybrids, starting with labeled and unlabeled homodimers. The hybrid isozymes were isolated by ion-exchange chromatography, and spectral analyses of singly labeled heterodimers revealed overlap between the absorption spectrum of MB labeled with acetamidofluorescein on the B subunit and the corrected fluorescence emission spectrum of MB labeled with CPM on the M subunit. Analyses included evaluation of the quantum yield of the CPM-labeled hybrid, estimation of the range of the orientation factor K^2 from fluorescence polarization and anisotropy studies, and determination of J, the spectral overlap integral for the fluorescence donor (CPM-labeled MB) and acceptor (acetamidofluorescein-labeled MB). Results of these experiments permitted an estimation of R_0, the distance between the donor and the acceptor at which energy transfer is 50% efficient. Comparison of the relative fluorescence of the donor in the presence (heterolabeled hybrid) and absence (hybrid conjugated with CPM on the M subunit) of the acceptor or determination of the normalized sensitization of the acceptor fluorescence led to an evaluation of the transfer efficiency and the actual transfer distance of between 27 and 52 Å. The kinetics of quenching of CPM fluorescence, during hybridization with CPM-labeled MM and acetamidofluorescein-labeled BB, compared to hybridization with unlabeled BB, suggest that substantial refolding of denatured subunits precedes reassociation.

Cytoplasmic creatine kinase occurs in three tissue-specific dimeric forms. The muscle isozyme, designated CK-MM,[1] has been extensively characterized both kinetically (Morrison & James, 1965; Jacobs & Kuby, 1970) and structurally (Yue et al., 1967). It is composed of two similar, if not identical, subunits of M_r 41 000, with an axial ratio of 4 for an assumed anhydrous prolate ellipsoid. Watts (1973) has likened the overall conformation to two cigar-shaped subunits positioned side by side, a conceptualization supported by X-ray crystallographic studies (McPherson, 1973). However, subunit association does not appear to be an obligatory requirement for catalytic activity (Grossman et al., 1981).

While the brain-type isozyme, CK-BB, is clearly distinct from the muscle type by the criteria of amino acid content (Watts, 1973; Grossman & Mollo, 1979), electrophoretic

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[1] Abbreviations: CK, creatine kinase; MM, the muscle type isozyme of creatine kinase; BB, the brain type isozyme; MB, the hybrid, myocardial isozyme; 5′-IAF, 5′-(iodoacetamido)fluorescein; CPM, 3-(4-maleimidylphenyl)-7-(diethylamino)-4-methylcoumarin; M_c, M subunit conjugated with CPM; B_f, B subunit conjugated with acetamidofluorescein; M_cM_c, B_fB_f, M_cB_f, M_cB, and MB_f, dye-conjugated dimers; DDT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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