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Subunit Dissociation of Mitochondrial Malate Dehydrogenase†

Joseph D. Shore* and Saroj K. Chakrabarti

ABSTRACT: Fluorescence polarization studies of porcine mitochondrial malate dehydrogenase labeled with fluorescein isothiocyanate or fluorescamine indicated a concentration-dependent dissociation of the dimeric molecule with a K_D of 2×10^{-7} N at pH 8.0. These results were confirmed by the concentration dependence of the stability of the enzyme at elevated temperatures and the creation of hybrid molecules with fluorescein and Rhodamine B labeled subunits, in which energy transfer was observed. The binding of NADH resulted in a small shift of the subunit dissociation curve toward monomer, demonstrating that monomer has twice the affinity for reduced coenzyme. NAD^+ binding prevented dissociation of the dimer, even at concentrations below 10^{-8} N. These results indicate that binding of reduced or oxidized coenzymes results in different conformation changes, which are transferred to the subunit interface.

Malate dehydrogenase (EC 1.1.1.37) from porcine heart mitochondria is a dimeric enzyme (Devenyi et al., 1966) with a molecular weight of 70 000 (Thorne and Kaplan, 1963), able to bind two molecules of NADH per dimer (Pfleiderer and Auricchio, 1964). Considering the anomalous kinetics reported for the enzyme (Harada and Wolfe, 1968) and our experience of instability of dilute solutions of the enzyme, the possibility of a monomer–dimer equilibrium was studied. The report of a monomer–dimer equilibrium for beef heart cytoplasmic malate dehydrogenase (Cassman and King, 1972) provided further interest in evaluating this possibility. Fluorescence techniques provided convenient and rapid methods for demonstrating and studying this equilibrium.

The polarization of fluorescence of a protein molecule covalently labeled with a fluorescent dye provides a sensitive method for the determination of rotational relaxation times (Weber, 1953), which would be expected to change with the degree of association of enzyme subunits. It has also been reported that protein association can be studied by singlet energy transfer between species labeled with different chromophores (Gennis et al., 1972). These techniques can be used to demonstrate the equilibrium between monomer and dimer and to determine the effects of coenzyme binding.

In the present study, fluorescence polarization of mitochondrial malate dehydrogenase covalently labeled with fluorescent dyes was determined as a function of enzyme concentration in the presence and absence of coenzymes. In addition, hybrid dimeric enzyme was created using donor–acceptor dye pairs and was used to evaluate the effects of NADH binding on the subunit dissociation.

Materials and Methods

Coenzymes and substrates were purchased from Sigma Chemical Company and used without further purification. Pig heart mitochondrial malate dehydrogenase was purchased from Miles-Seravac (Batch 95 AB) and exhibited maximum fluorescence at 307 nm when excited at 280 nm, indicating the absence of tryptophan-containing proteins. Enzyme activity was determined by the method of Gregory et al. (1971). The concentration of active enzyme was determined by titration with NADH in the presence of hydroxymalonate at pH 6.4 by the method of Holbrook and Wolfe (1972).

The reagents used for fluorescence labeling of the enzyme were fluorescein isothiocyanate on Celite purchased from Calbiochem, Rhodamine B isothiocyanate purchased from Sigma Chemical Company, and fluorescamine purchased from Roche diagnostics. Fluorescein-labeled enzyme was prepared by adding 1 mg of fluorescein isothiocyanate on Celite to 4 ml of 30 μN enzyme in 0.05 M (pH 8.0) Tris-acetate buffer and stirring for 0.5 h at 4 °C. The amount of fluorescein labeling was determined by the absorbance at 490 nm, using an extinction coefficient of 3.4×10^4 $l.^{-1}$ M cm^{-1} (Churchich, 1967). The preparations consistently

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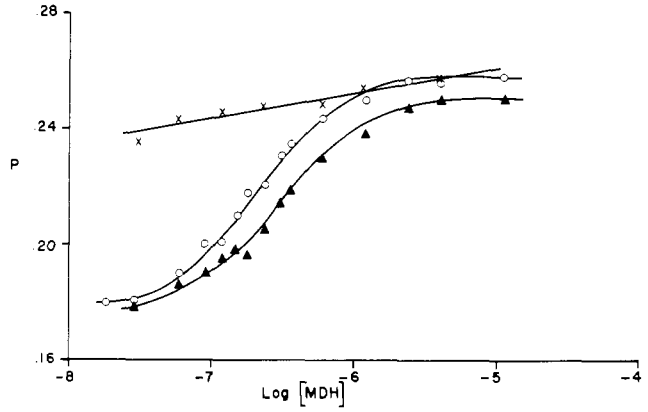

FIGURE 1: Fluorescence polarization vs. log of fluorescein-labeled MDH concentration. (○) Free enzyme; (▲) enzyme in presence of 25 μM NADH; (×) enzyme in presence of 10 mM NAD^+. Excitation, 490 nm; emission, 520 nm.

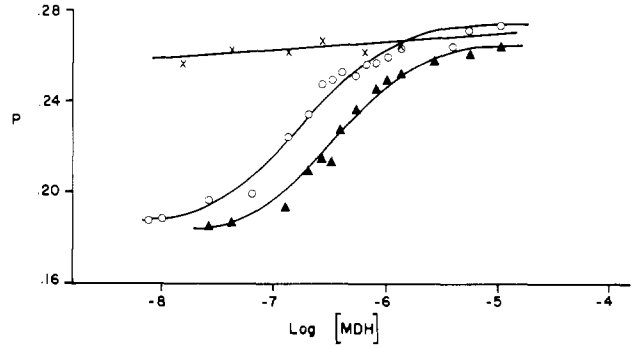

FIGURE 2: Fluorescence polarization vs. log of fluorescamine-labeled MDH concentration. (○) Free enzyme; (▲) enzyme in presence of 25 μM NADH; (×) enzyme in presence of 10 mM NAD^+. Excitation, 395 nm; emission 480 nm.

contained 0.5 ± 0.2 fluorescein molecule per equivalent of enzyme. Although it was possible, using higher fluorescein and enzyme concentrations, to obtain a product with as many as two fluorescein molecules per enzyme equivalent without activity loss, care was taken to not do this since the fluorescence polarization measurements would be distorted by energy transfer between chromophores. Rhodamine B labeling was carried out by adding 0.3 ml of a 0.5 mg/ml solution in the isothiocyanate in acetone to 4 ml of 30 μN enzyme in 0.05 M (pH 8) Tris-acetate buffer, followed by stirring for 2 h at 4 °C. The degree of labeling with Rhodamine B was determined by the absorbance at 550 nm, using an extinction coefficient of 1.23×10^4 $l.^{-1}$ M cm^{-1} (Chen, 1969). The preparations obtained contained 2 ± 0.5 Rhodamine B molecules per equivalent of enzyme. The higher degree of labeling with Rhodamine B as compared to fluorescein facilitated energy transfer studies since the fluorescence yield of the fluorescein-labeled enzyme is considerably higher. Fluorescamine-labeled enzyme was prepared by adding 0.5 ml of a 1 mM solution of dye in acetone to 4 ml of 30 μN enzyme in 0.2 M (pH 9.0) borate buffer. Subsequent to labeling with all three reagents the enzyme was separated from free label by Sephadex G-25 chromatography. Activity determinations indicated that less than 10% of the enzyme activity had been lost due to labeling.

Fluorescence spectra were obtained with a Farrand spectrofluorimeter, and are not corrected for variations in lamp intensity or photomultiplier sensitivity with wavelength. Fluorescence polarization was determined with the Farrand instrument, using Polacoat filters. Polarization was calculated using the equation $P = (\| - G\perp)/(\| + G\perp)$, where $\|$ represents vertical excitation and emission polarizer, $\perp$ represents a vertically polarized excitation and horizontally polarized emission, and G represents a correction factor consisting of the ratio of vertical to horizontally polarized emission with excitation in the horizontal plane (Azumi and McGlynn, 1962). The precision of the polarization measurements was ±2% of the measured value at the lowest concentrations studied. All studies were performed at 23 °C in 0.05 *M* Tris-acetate buffer at pH 8.0.

Results

The polarization of fluorescence of fluorescein-labeled enzyme was determined at various concentrations. A plot of polarization vs. concentration of enzyme subunits is given in Figure 1. As the enzyme was diluted the polarization fell from a level of 0.26 at 11 μN enzyme to 0.18 at 0.018 μN enzyme, the lowest concentration studied. In the presence of 25 μM NADH, the curve was shifted toward lower polarization values, but in the presence of 10 mM NAD^+ the enzyme remains in a form resulting in a high fluorescence polarization. The gradual slope of the line in the presence of 10 mM NAD^+ is a real phenomenon and most probably due to the fact that, with a dissociation constant of 0.5 mM for NAD^+ (Holbrook and Wolfe, 1972), the enzyme is only 95% saturated with oxidized coenzyme. Since both NAD^+ and NADH absorb and emit light in a wavelength region far from the fluorescein signal being monitored, the observed changes can be assumed to be due to properties of the labeled enzyme rather than the coenzymes.

In order to reaffirm the phenomenon, the experiment was repeated using fluorescamine-labeled enzyme. The results (Figure 2) were identical with those obtained using fluorescein-labeled enzyme. Dilution of the enzyme from a concentration of 10^{-5} to 7.6×10^{-9} N resulted in a decrease in fluorescence polarization from 0.27 to 0.19. In the presence of 10 mM NAD^+ the polarization change due to dilution was slight, while in the presence of 25 μM NADH the curve was shifted to the right. Although there is some overlap between the emission spectra of fluorescamine-labeled enzyme and NADH, the quantum yield of the fluorescamine-labeled enzyme was so much higher than that of the reduced coenzyme that the contribution of NADH to the polarization signal was negligible at the instrument sensitivity used.

Since changed polarization could be due to either a changed rotational relaxation time or an altered fluorescence lifetime, the lifetime of the fluorescein-labeled enzyme was determined at concentrations of 10^{-5} and 5×10^{-8} N using a TRW nanosecond fluorimeter and a Tektronix Model 5504 sampling oscilloscope. The lifetimes of both samples were 5 ± 0.5 ns, indicating that the changed polarization due to dilution was not the result of a changed lifetime of the fluorescein-labeled enzyme.

The most reasonable explanation for a decreased rotational relaxation time due to dilution of the enzyme would be dissociation of the dimeric enzyme to monomers. Since the native state of the enzyme is a dimer, it seemed feasible that the monomer would be less stable than the dimer. Consequently, the inactivation of the enzyme by elevated temperature was determined at 4×10^{-6} and 1.5×10^{-7} N enzyme concentrations. These experiments were performed using native enzyme, which had not been labeled with fluorescent dyes. The results (Figure 3) demonstrated that the stability of the enzyme at 30 °C is very dependent on en-

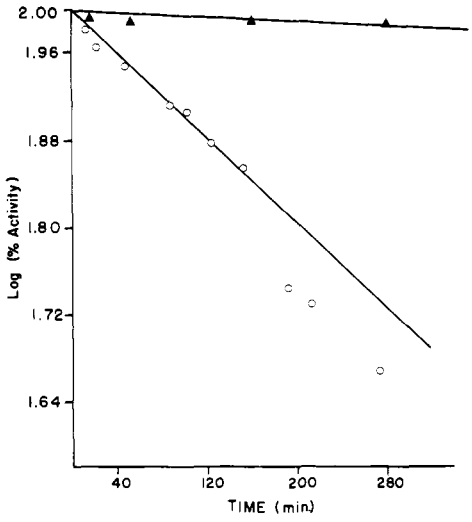

FIGURE 3. Stability of malate dehydrogenase at 30 °C. (▲) 4.0 μN enzyme; (O) 0.15 μN enzyme.

zyme concentration. In 4 h almost no activity loss occurred in the concentrated solution while the dilute enzyme became 50% inactivated.

The existence of a monomer-dimer equilibrium should enable creation of hybrid molecules in which each subunit is labeled with a different dye, by mixing concentrated solutions of enzyme covalently labeled with the two dyes. Although it is not possible to isolate the hybrid molecules, a proportion of the molecules in the mixture will be hybrids, depending on the molar ratio of the labeled species. If a suitable dye pair is chosen, with the emission spectrum of the donor overlapping the excitation spectrum of the acceptor, the population of hybrid molecules will provide a convenient signal for monitoring subunit dissociation by singlet-singlet energy transfer. The fluorescent dye pair chosen for hybrid formation was fluorescein and Rhodamine B. Figure 4 shows the emission spectrum of a mixture of 2 μN fluorescein-labeled enzyme and 6 μN Rhodamine-labeled enzyme, excited at 490 nm. For comparison, the emission spectrum of the same concentration of fluorescein-labeled enzyme was included. The Rhodamine B labeled enzyme showed no fluorescence emission in the 550-600-nm region with 490-nm exciting light at the instrument sensitivity used. It can be seen that the fluorescence of the fluorescein-labeled enzyme is quenched in the mixture, with the concomitant appearance of a peak at 580 nm. The relatively small effect can be accounted for by the low red response of the 1P-28 photomultiplier tube, the presence of a dimer species containing fluorescein on both subunits, the substantially higher quantum yield of fluorescein, and the distance between the chromophores on the molecules of hybrid dimer.

Excitation spectra and polarization excitation spectra were also made with the hybrid mixture, using emission at 590 nm. The results, shown in Figure 5, indicate that energy transfer has occurred in the mixture. The appearance of a double peak in the mixture containing hybrid, and the polarization value of 0.145 at 490 nm compared with 0.26 for comparable concentrations of fluorescein-labeled enzyme with emission measured at 520 nm, demonstrate the existence of energy transfer. Between 490 and 540 nm, the polarization rises to a new level since the chromophore being observed changes from fluorescein to Rhodamine B, with the level observed below 540 nm being a function of both

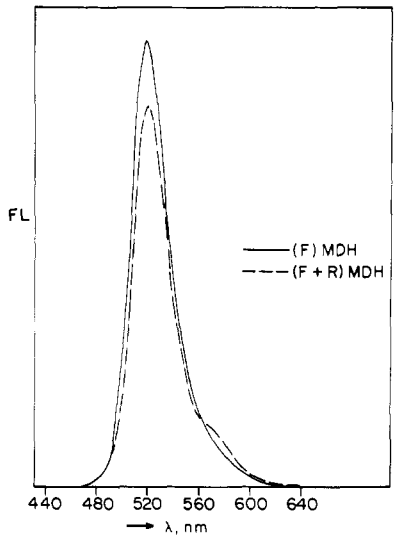

FIGURE 4: Emission spectra of fluorescein-labeled MDH and the fluorescein-Rhodamine B hybrid with excitation at 470 nm. (—) 2 μN fluorescein labeled enzyme; (- - -) 2 μN fluorescein-labeled enzyme in the presence of 6 μN Rhodamine B labeled enzyme.

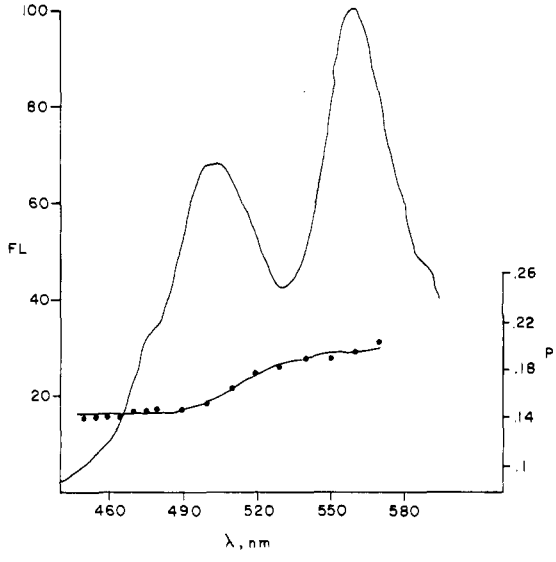

FIGURE 5: Excitation (—) and fluorescence polarization excitation (•—•) spectra of the fluorescein-Rhodamine B hybrid MDH. The mixture contained 1.5 μN fluorescein-labeled enzyme and 4.5 μN Rhodamine B labeled enzyme. Emission was measured at 595 nm.

the apparent lifetime of the fluorescein and the orientation between the donor and acceptor molecules. At the concentrations of enzyme used, it can be assumed that only dimers are present.

The dissociation of a hybrid enzyme dimer into its components should result in loss of energy transfer since the distance between the subunits will be too great. This is an all or none phenomenon since only those subunits in a dimeric state can transfer energy. Consequently, excitation of the donor with measurement of the sensitized fluorescence of the acceptor provides a convenient signal for measuring subunit dissociation. The data presented in Figures 1 and 2 indicate that NADH binding results in a shift of the monomer-dimer equilibrium in the direction of greater dissociation. This effect was monitored by titrating the mixture containing fluorescein-Rhodamine B labeled hybrid with NADH (Figure 6). The fluorescence was monitored using

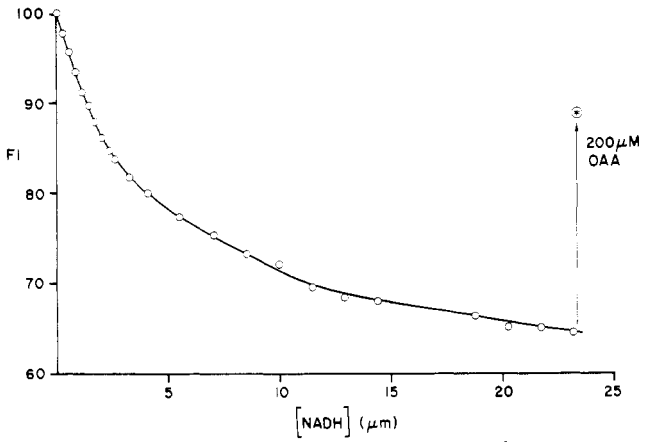

FIGURE 6: Titration of fluorescein–Rhodamine B hybrid MDH with NADH. The mixture contained 1.5 μN fluorescein-labeled enzyme and 4.5 μN Rhodamine B labeled enzyme. Excitation, 490 nm, emission, 590 nm.

excitation at 490 nm and emission at 590 nm, with the signal expanded to give a relative fluorescence value of 100. Addition of reduced coenzyme resulted in loss of energy transfer, although a constant level was not attained as would be expected from the reported (Holbrook and Wolfe, 1972) hyperbolic binding of NADH. At the end of the titration oxaloacetate was added resulting in a return of the energy transfer level to 90%. The failure to return to 100%, and continual gradual sloping above 10 μM NADH, are most probably due to inactivation of monomeric species as the titration progressed. Since the wavelengths used in this titration were quite far from the excitation peak of the NADH at 340 nm, the changes observed in the transfer signal must be due to the dye-labeled enzyme.

Discussion

Past studies, using ultracentrifugation (Wolfe and Neilands, 1956) and gel filtration (Pfleiderer and Auricchio, 1964), indicated that the molecular weight of pig heart mitochondrial malate dehydrogenase is approximately 70 000. It has also been established (Pfleiderer and Auricchio, 1964, Holbrook and Wolfe, 1972) that the enzyme binds two molecules of NADH per mole. Peptide fingerprinting (Devenyi et al., 1966) demonstrated that the enzyme contains two subunits of identical composition. Since these physical methods used for molecular weight determination were performed at high enzyme concentration, the enzyme form showing a higher level of fluorescence polarization represents the dimeric species.

The dissociation of the dimer to monomer in our studies is indicated by several types of evidence. The decrease in fluorescence polarization due to dilution, with an unchanged lifetime of the fluorescein-labeled enzyme, indicates a diminished average rotational relaxation time. The possibility that the polarization changes observed with fluorescein-labeled enzyme are due to noncovalently bound dye dissociating from the enzyme upon dilution has been considered. Extensive dialysis of labeled enzyme, and rechromatography on Sephadex G-25, did not change the polarization levels or the dilution effect. This possibility can also be ruled out by the results of Figure 2 with fluorescamine-labeled enzyme, since this dye does not fluoresce unless it has covalently reacted with a primary amino group. Another possible artefact would be the presence of inactive enzyme molecules which can dissociate. However, the almost complete activity recovery after labeling, which was frequently 100%, would tend to rule out this possibility. The curves obtained in Figures 1 and 2 correspond to a dissociation constant of approximately 2×10^{-7} N for the dimeric enzyme.

In studies currently in progress, we have been able to regenerate the dimer polarization level by adding an excess of unlabeled enzyme to 10^{-7} N labeled enzyme. This would indicate that the dissociating species is not an inactive protein, or is at least able to exchange with active enzyme subunits. It also provides evidence that the dissociation phenomenon is not a unique characteristic of labeled enzyme, as do the heat inactivation data. The results of Figure 3 indicate that the monomer is less stable to heat inactivation than the dimer, and provide further evidence for dimer dissociation since 10^{-7} N enzyme was inactivated much more rapidly than 4×10^{-6} N enzyme. Regarding the possibility of the heat inactivation results being due to surface denaturation, the concentration of dilute enzyme in Figure 3, 10^{-7} N, is considerably higher than usually reported for surface denaturation. Furthermore, the time dependence of activity loss and stability of 10^{-7} N solutions at lower temperatures would argue against a major role of surface effects in the inactivation. Hybrid formation with fluorescein and Rhodamine B labeled enzymes also substantiates the existence of a dimer–monomer equilibrium.

It is impossible to accurately calculate the expected polarization values for the malate dehydrogenase monomer and dimer without making assumptions regarding specificity of labeling, molecular shape, degree of hydration, and flexibility of the molecule. However, a model calculation assuming a P_0 value of 0.45 for fluorescein dimer and monomer, spherical shape for both with dimer having twice the volume of monomer, and a polarization of 0.3 for dimer results in a calculated value of 0.22 for the polarization of monomer. The results obtained are thus not incompatible with a dimer dissociation, although the approximate nature of these calculations must be recognized.

The intramitochondrial concentration of malate dehydrogenase is high enough to ensure that the enzyme is a dimer in vivo. Nevertheless, the phenomenon of subunit dissociation in dilute solution provides a useful probe of both the effects of ligands on conformation and interaction between subunits. The decreased subunit dissociation of dimer due to NAD^+ binding and shift in the dissociation curve due to NADH binding indicate that both ligands are causing conformational changes. Since the effects are different, oxidized and reduced coenzyme must be causing different conformational changes. Furthermore, since both ligands affect the equilibrium between dimer and monomer, the conformational changes are transferred to the subunit interface. Although it is a likely possibility, these studies do not conclusively demonstrate that the conformational changes due to coenzyme binding affect the binding or kinetic properties of the active site on the adjacent subunit.

The titration of fluorescein–Rhodamine B hybrid enzyme with NADH, resulting in a decrease of sensitized fluorescence, further supports the shift toward monomer indicated by the fluorescence polarization studies of Figures 1 and 2. The results obtained in Figure 6 indicate a dissociation constant for NADH of the same general magnitude as that reported by Holbrook and Wolfe (1972). It is necessary to reconcile the hyperbolic binding reported for reduced coenzyme in that study with the subunit dissociation. Since the shift in the subunit dissociation constant due to NADH

binding is approximately twofold (Figures 1 and 2), this would indicate that monomer binds reduced coenzyme with twice the affinity of dimer. A fluorimetric study of NADH binding to the enzyme, performed at enzyme concentrations under which the enzyme was primarily in the dimeric form even after coenzyme was bound, could not discern a twofold difference in dissociation constants. On the other hand, oxidized coenzyme binding at low enzyme concentrations should show marked effects, since the results of Figures 1 and 2 indicate that the dimer binds NAD^+ at least an order of magnitude more tightly than the monomer. If the dissociation constant of a monomer–dimer equilibrium is affected by coenzyme binding, the two processes must be thermodynamically linked (Ackers and Halvorson, 1974). Regardless of the pathway, the affinity of NAD^+ for dimer will be much greater than for monomer. This aspect of the enzyme reaction mechanism is currently being investigated.

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Lipoprotein Lipase: Evidence for High- and Low-Affinity Enzyme Sites

C. J. Fielding

ABSTRACT: The kinetic constants for membrane-supported lipoprotein lipase have been determined for the enzyme active in lipoprotein triglyceride catabolism in perfused heart and adipose tissues, using a nonrecirculating system. Heart endothelial lipoprotein lipase reacted as a single population of high-affinity substrate binding sites (K_m' 0.07 mM triglyceride). K_m' (apparent Michaelis constant for the supported enzyme species) was independent of flow rate and the enzyme was rapidly released by heparin, suggestive of a superficial membrane binding site. Lipoprotein lipase active in perfused adipose tissue had significantly different kinetic properties, including a low substrate affinity (K_m' 0.70 mM triglyceride), diffusion dependence of K_m' at low flow rates, and slow release of enzyme by heparin. Adipose tissue may contain a small proportion of high affinity sites. While only a small proportion of total heart tissue lipoprotein lipase was directly active in triglyceride hydrolysis, this study suggests that the major part of lipoprotein lipase in adipose tissue may be involved in the hydrolysis of circulating lipoprotein triglyceride.

In a previous study (Fielding and Higgins, 1974) it was shown that the kinetic activity of membrane-supported lipoprotein lipase, based on a theoretical treatment for flow through an enzyme column (Lilly et al., 1966) followed predicted Michaelis–Menten kinetics for several lipoprotein substrates in a recirculating perfusion system. Such techniques provide a means of investigating the behavior of membrane–enzyme complexes whose lability has precluded their isolation, as is the case for the lipoprotein lipase system. They can also be used to determine the effect of the support environment, for example its electrical charge, on the kinetic characteristics of enzyme systems (Goldstein, 1972). Although the lipoprotein lipase system was apparently the first in which a natural membrane system was studied in this way, the techniques had been previously validated for several systems of soluble enzymes which had been immobilized on cellulose or polyacrylamide supports (Lilly et al., 1966; Goldman et al., 1971; Bunting and Laidler, 1972). It can be shown that, at low flow rates, while maximum reaction velocity is unchanged, the apparent Michaelis constant for the membrane-associated enzyme species becomes flow dependent below a limiting rate (Hornby et al., 1968; Sundaram et al., 1970). The apparent K_m'[1] value is dependent upon the rate constant of the en-

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[1] Abbreviations used in this paper are: S_f, flotation index at solvent density 1.063 g/ml; V_{max}', V_{max}, maximal reaction velocities for the supported and soluble enzyme species; K_m', K_m, apparent Michaelis constants for the supported and soluble enzyme species.