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Translational Mobility of Glycophorin in Bilayer Membranes of Dimyristoylphosphatidylcholine†

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ABSTRACT: The translational diffusion of the integral membrane sialoglycoprotein from erythrocyte membranes, glycophorin, incorporated into bilayer membranes of dimyristoylphosphatidylcholine at a protein/lipid molar ratio of 1:4500 was examined by using the fluorescence redistribution after photobleaching technique. A plot of the diffusion coefficient vs. temperature shows a sharp decrease in the rate of diffusion at about 15 °C. This sharp diffusion transition is at a temperature some 9 °C lower than the calorimetrically measured lipid gel–liquid crystalline phase transition temperature of the system. The difference between the diffusion transition temperature and the lipid phase transition temperature is attributed to a localized fluidizing effect of the protein upon the gel phase lipid. The value of the diffusion coefficient above 15 °C was found to be (1–2) × 10^{-8} cm^2 s^{-1}, and below 15 °C it was lower than about 5 × 10^{-11} cm^2 s^{-1}. The fluorescence recovery in the bleached area as a consequence of diffusional redistribution appeared to be due to a single diffusing species at temperatures above 15 °C and due to more than one diffusing species below this temperature.

The translational and rotational diffusion of membrane components is of considerable biochemical and biophysical interest [for a recent review, see Cherry, (1979)]. One approach to the understanding of diffusion in biological membranes is the study of diffusion in "reconstituted" model membranes. In such systems, with a limited number of well-defined components, the interactions of the components with each other and the behavior of the system as a whole are generally fairly well understood. The study of diffusion of membrane-bound proteins in simple model systems could yield important insights into the problem of diffusion of membrane-bound proteins in the far more complex biological membranes. Further, an experimental evaluation of existing theoretical treatments (Saffman & Delbrück, 1975; Saffman, 1976) of diffusion in quasi-two-dimensional systems such as membranes may be useful.

The experimental study of the translational diffusion of proteins in reconstituted membranes is fairly new (Derzko & Jacobson, 1978; Vaz et al, 1979a,b; Smith et al., 1979a; Schindler et al., 1980). These studies have been made possible

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0006-2960/81/0420-1392$01.25/0

since the theoretical and practical description of the FRAP[1] technique (Axelrod et al., 1976). In view of its simplicity, this technique is particularly attractive for the study of translational diffusion over long ranges (several micrometers) in membranes. This paper reports our results on the translational diffusion of glycophorin in bilayer membranes of DMPC. Glycophorin is the major sialoglycoprotein of erythrocyte membranes and is one of the best characterized integral membrane proteins (Romans et al., 1978; Marchesi, 1978). It has a monomeric molecular weight of about 31 000, of which about 40% is due to the peptide portion, the rest being due to the sugar residues. In the native membrane (Romans et al., 1978; Marchesi, 1978) as well as in reconstituted systems (van Zoelen et al., 1978), the protein is known to have a trans-membrane orientation with an apolar membrane-penetrating peptide section having a molecular weight of about 2500.

Materials and Methods

Outdated blood was obtained from the blood bank of the University Clinic, Göttingen. Erythrocyte ghosts were prepared by osmolysis (Dodge et al., 1963). Glycophorin was extracted from the erythrocyte ghosts by using a detergent extraction procedure (Segrest et al., 1979) which has the advantage over the older lithium diiodosalicylate extraction method (Marchesi & Andrews, 1971) in that purified glycophorin is obtainable in a form that is free from the detergent and lithium diiodosalicylate (Segrest et al., 1979). The purified glycophorin was stored at −70 °C as an aqueous solution containing 5 mg of glycophorin per mL. Glycophorin was labeled with fluorescein by reaction with a 100 times molar excess of fluorescein 5′-isothiocyanate (Molecular Probes Inc., Plano, Tx) in 0.1 M sodium borate, pH 9.0, at 4 °C for 4 h in the dark. After reaction, the unreacted reagent was separated from labeled protein by filtration through Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). It was found to be necessary to filter the labeled protein twice through Sephadex G-25 to remove all traces of noncovalently bound dye. After filtration, the labeled glycophorin was concentrated to 5 mg/mL. The dye/protein molar ratio of the product was about 1.0. DMPC was a product of Fluka AG, Buchs, Switzerland, and was checked to be free of lysophosphatidylcholine and free fatty acids by thin-layer chromatography.

The reconstitution of glycophorin into DMPC bilayer membranes followed essentially the method described by MacDonald & MacDonald (1975): 225 parts of a 2:1 mixture of chloroform and methanol containing the DMPC at 40 °C were mixed with 1 part of a solution of labeled glycophorin in distilled water (5 mg/mL) also at 40 °C. The solvent was evaporated at 40–50 °C by rotary evaporation in a round-bottomed flask, and the resulting residue was dried overnight at 40 °C. This residue was then hydrated at 40 °C with distilled water. After hydration, the turbid suspension was centrifuged at 10000*g* for 30 min at 25 °C. The pellet was resuspended in a desired volume of distilled water for further use, and the supernatant was discarded. Glycophorin–DMPC proteoliposomes prepared in this manner were used for differential scanning calorimetry using a Perkin-Elmer DSC-2 scanning calorimeter. The protein/lipid molar ratio was 1:4500 in all cases.

Slides for FRAP studies were prepared from the resuspended 10000*g* proteoliposome pellet as follows: A 0.1-mL aliquot of the suspension containing 2 mg of lipid was deposited on a clean glass slide and allowed to dehydrate overnight at 40 °C in water-saturated air. The partially dehydrated film was brought back to conditions of excess water by dropping a cover slip over the residue with a hanging drop of 20 μL of Dulbecco's phosphate-buffered saline free of Ca^{2+} and Mg^{2+} ions which had been diluted 10 times with distilled water. The slide was then sealed and left overnight at 40 °C before use. At the end of this incubation period the hydrated lipid residue appeared as a jumbled mass at the edges of which large spherical (40–70-μm diameter) liposomes had formed. It was not possible to ascertain the exact number of lamella constituting these liposomes. In some, the fluorescence derived from the incorporated labeled protein was more pronounced at the circumference than in the center, as would be consistent with a uni- or paucilamellar structure. Other liposomes showed a uniform fluorescence throughout and were assumed to be multilamellar. Some large, apparently paucilamellar, liposomes seemed to include smaller liposomes within them. These last type of structures were avoided in the FRAP experiments. Figure 1 shows some typical liposomes used for FRAP studies. No differences in the fluorescence recovery curves were observed between apparently paucilamellar and multilamellar liposomes.

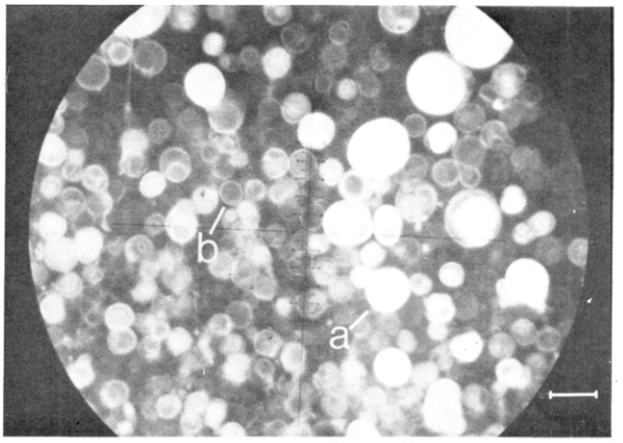

FIGURE 1: Fluorescence micrograph of a typical preparation of DMPC–glycophorin proteoliposomes used in the FRAP studies. Apparently multilamellar liposomes (a) and uni- or paucilamellar liposomes (b) are indicated. The inserted bar indicates 60 μm.

FRAP experiments were performed as described by Kapitza & Sackmann (Kapitza, 1979; Kapitza & Sackmann, 1980) on the microscope fluorimeter described by these authors except that in this study a Zeiss Planapo 25 ×/0.65 Pol objective was used which resulted in a circular observation area of 11-μm diameter. An argon ion laser (Spectra Physics, Model 165-04) was used for photobleaching at 488 nm. The incident power at the slide in a bleaching pulse was 1.5 mW. Bleaching pulse times were $^1/_{16}$–$^1/_8$ s at temperatures above 20 °C where the fluorescence recovery times were fast (3–7 s half-times) and $^1/_4$ s below 20 °C where the fluorescence recovery was slow (half-times of several minutes). Diffusion coefficients were calculated from the fluorescence recovery curves by using the expression (Axelrod et al., 1976) for an uniform circular beam profile:

$$D_T = \frac{0.88w^2}{4t_{1/2}}$$

where w is the radius of the circular observation area and $t_{1/2}$ is the time required for the fluorescence intensity in the bleached area to reach 50% of its intensity after complete redistribution of fluorescence. The ratio of fluorescence intensity at "infinite" time after photobleaching to the intensity

[1] Abbreviations used: FRAP, fluorescence redistribution after photobleaching; DMPC, dimyristoylphosphatidylcholine.

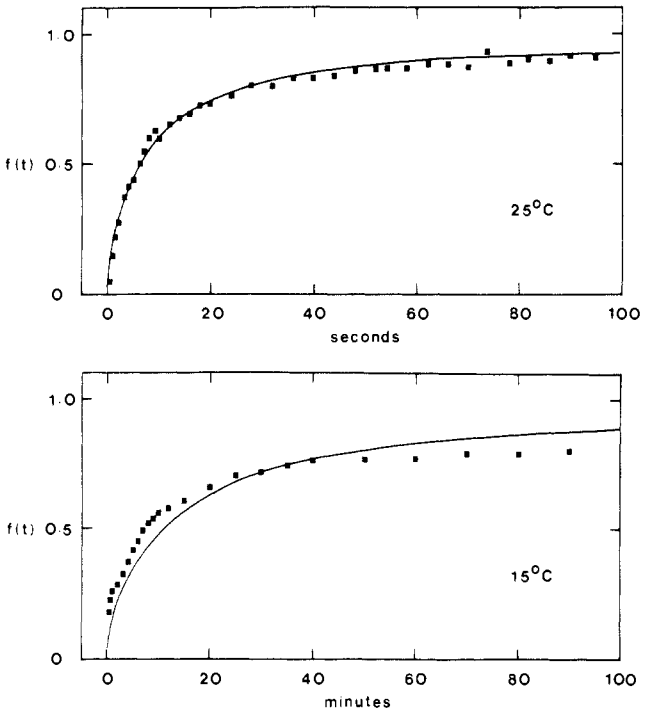

FIGURE 2: Recovery of fluorescence after photobleaching at 25 °C (upper panel) and at 15 °C (lower panel). The curves are plotted as fractional fluorescence recoveries, as defined by eq 9 of Axelrod et al. (1976), vs. time. The points are experimental values and the lines are theoretical recovery curves for fractional fluorescence recovery after photobleaching due to redistribution of a single diffusing species. The theoretical curves were calculated by numerical evaluation using eq 14 of Axelrod et al. (1976).

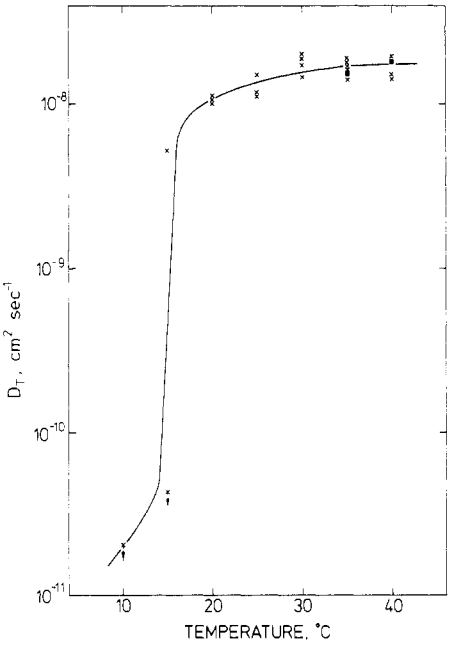

FIGURE 3: Plot of the translational diffusion coefficient vs. temperature for diffusion of glycophorin in DMPC bilayer membranes. The lipid/protein molar ratio was 4500:1. The arrows below the points at 15 and 10 °C are to indicate that these values represent upper limits for the diffusion coefficient at these temperatures. A 30-min thermal equilibration time was allowed at each temperature. Each point is the mean value obtained from between three and five FRAP experiments on the same sample. The solid squares indicate FRAP experiments on a liposome preparation which had been studied in a temperature scan from 40 through 10 °C and then raised back to the temperature of the experimental point.

before photobleaching was 0.85–0.98 depending upon the type of liposome under study.

Results

Reconstitution of Glycophorin in DMPC Membranes. The reconstitution of glycophorin into DMPC membranes using the method of MacDonald & MacDonald (1975) apparently does not result in association of all of the added glycophorin with the lipid. After centrifugation of the hydrated proteoliposomes we noted that there was always some nonpelletable glycophorin in the supernatant and that this was the case even upon centrifugation at 100000*g* for 60 min. This suggests that the nonsedimentable glycophorin was either not associated with lipid or, if associated, that the protein–lipid complexes formed were of a relatively small size. Other workers have recently shown (MacDonald, 1980) that the reconstitution procedure used results in about 30% of the added glycophorin being not associated with lipid when the entire reconstitution procedure is carried out at a temperature above the lipid-phase transition temperature. The amount of non lipid-associated protein was found to be even greater when the reconstitution was done below the lipid phase transition temperature. In FRAP studies it is important to be sure that all of the protein in the sample is associated with the lipid in order to avoid apparent fluorescence recoveries resulting from aqueous diffusion of nonlipid-associated protein. Nonassociated protein would be evident in the slides used for FRAP experiments as fluorescence in the aqueous lipid-free zones of the slide. No such fluorescence was observed, indicating that all of the glycophorin was lipid associated.

FRAP Results. Typical curves for the recovery of fluorescence in a photobleached area are shown in Figure 2. As seen, the experimental fluorescence recovery curve at 25 °C fits a theoretical curve for fluorescence recovery due to redistribution of a single diffusing species quite well. This was found to be generally true for all fluorescence recovery curves above 15 °C. At 15 °C and below, however, this was no longer observed to be the case. A similar situation has been reported recently for lipid probe diffusion in gel-phase phosphatidylcholine multibilayers (Derzko & Jacobson, 1980). In the case of the lipid probes, the multicomponent recovery of fluorescence after photobleaching has been attributed to the existence of structural defects in the gel phase lipid bilayers (Derzko & Jacobson, 1980). In all our experiments the fluorescence intensity immediately after photobleaching was between 35 and 65% of the fluorescence intensity prior to photobleaching. Also, the recovery of fluorescence at "infinite" times after the photobleaching event was 85–98% of the intensity prior to photobleaching. The incomplete recoveries were at least partially due to depletion of the fluorescent labels during photobleaching. Visual observation as well as scanning the monitoring beam across a proteoliposome after complete redistribution of fluorescence following photobleaching indicated no inhomogeneities in the fluorescence intensity across the liposomes. A plot of the translational diffusion coefficients for glycophorin in DMPC liposomes as a function of temperature is shown in Figure 3. The fluorescence remained uniformly distributed throughout the liposomes at all temperatures down to 5 °C. This is in contrast to results with the M-13 viral coat peptide in DMPC multibilayers and liposomes (Smith et al., 1979a). We conclude that domains of extensive aggregation as seen with the M-13 viral coat peptide do not occur with glycophorin in DMPC bilayers below the lipid phase transition temperature. The translational diffusion of glycophorin in DMPC membranes in the lipid fluid state is somewhat slower than the translational diffusion of lipid probes (Wu et al., 1977; Fahey & Webb, 1978; Kapitza, 1979), small hydrophobic peptides (Wu et al., 1978), the membrane-

spanning M-13 viral coat peptide (Smith et al., 1979a), and an apolipoprotein which binds to the head-group region of the phospholipid bilayer (Vaz et al., 1979a,b), all measured in fluid-state lipid bilayers. A sharp decrease in the diffusion rate (represented by a drop in the translational diffusion coefficient by a factor of about 200) is seen in a relatively narrow temperature range around 15 °C. The magnitude of the slowing of glycophorin diffusion is of the same order as the slowing of lipid probe diffusion in the lipid gel state compared with the lipid fluid state (Wu et al., 1977, 1978; Fahey & Webb, 1978; Kapitza, 1979). However, these latter entities show a drop in the diffusion rate at a temperature corresponding almost exactly with the phase transition temperature of the host lipid whereas the slowing of glycophorin diffusion occurs some 9 °C below the transition temperature of the host lipid. It must be noted that the phase transition behavior of the glycophorin-containing lipid bilayers as studied by us is not significantly different from that of the protein-free lipid bilayer measured by differential scanning calorimetry (data not shown). Similar behavior was seen in the case of Apo C-III diffusion in multibilayers of dipalmitoylphosphatidylcholine (Vaz et al., 1979a,b).

Discussion

The relatively fast translational diffusion of glycophorin in the fluid state lipid bilayer is in agreement with reports on the diffusion of other membrane-bound proteins in model membranes (Derzko & Jacobson, 1978; Smith et al., 1979a; Vaz et al., 1979a,b), but is several times faster than that observed for integral membrane proteins in natural (plasma) membranes [see, for example, Cherry (1979)]. Several mechanisms have been proposed to explain the relatively slow translational diffusion of integral membrane proteins in natural membranes (Edelman, 1976; Nicolson, 1976). These include association of membrane components within the bilayer and interactions of the integral proteins with cytoskeletal and extramembranous organized structures. In model membranes it has been demonstrated that cholesterol significantly reduces the translational mobility of a membrane-bound protein (Vaz et al., 1979a,b). Studies on reconstituted model membranes of increasing complexity and comparison with parallel studies on natural membranes may be one way to resolve these mechanisms.

Interestingly, and also in agreement with previous studies (Vaz et al., 1979a,b), the protein embedded in the lipid demonstrates a transition, as reflected in the values of the translational diffusion coefficient, at a temperature about 9 °C lower than that of the lipid phase transition. We suggest that this may be a sort of "ice-breaker" effect where the protein disorders the gel phase lipid in a certain area around itself to a sufficient degree as to diffuse in a bulk gel phase lipid bilayer as though it were in a relatively fluid environment. Such a result was predicted in the specific case of glycophorin on the basis of ^{13}C nuclear magnetic resonance studies on glycophorin-containing phosphatidylcholine bilayers (Brûlet & McConnell, 1976). A local disordering of gel phase lipid around a protein molecule in a bilayer would not be detected by calorimetric studies when the ratio of lipid/protein is high, as is the case in this study. Significant depression of the lipid phase transition temperature and transition enthalpy is observed only at low lipid/protein ratios (W. Vaz, unpublished results).

Another interesting aspect is the influence of the large hydrophilic portions of glycophorin upon the translational diffusion of this protein in a lipid bilayer. This could be verified in the case of glycophorin by examining the diffusion of the hydrophobic tryptic fragment of this protein (Segrest et al., 1974) in reconstituted systems. Such studies are currently being done by us. However, on the basis of the results of other workers (Smith et al., 1979b), one may not expect any significant differences between the translational diffusion rates of glycophorin and its hydrophobic tryptic fragment.

A comparison of the translational diffusion rates of glycophorin and the M-13 viral coat peptide, both of which have membrane-bound segments of approximately the same size, is of interest. The value of the diffusion coefficient reported for the M-13 viral coat peptide at 30 °C in DMPC bilayers was 4×10^{-8} cm^2 s^{-1} (Smith et al., 1979a). For glycophorin in DMPC bilayers at the same temperature our value is about 2×10^{-8} cm^2 s^{-1}, i.e., a factor of about 2 slower than that seen for the viral coat peptide. Given the known experimental uncertainties in the photobleaching technique, the observed difference would be significant only if determined with the same apparatus. However, we cannot exclude that the diffusion coefficient for glycophorin is depressed due to either the massive hydrophilic segments and/or aggregation of the protein within the lipid bilayer. An aggregation size of about 6–7 monomeric units has been proposed for glycophorin in a membranous environment (Romans et al., 1978). Existing theory for the translational diffusion of integral membrane proteins spanning the membrane (Saffman & Delbrück, 1975; Saffman, 1976; Galla et al., 1979) predicts values compatible with the present measurements. However, the dependence upon particle size (or radius) is weak.

The rotational diffusion of glycophorin reconstituted in DMPC vesicles has been examined as well by using the polarized phosphorescence decay of an external triplet probe (M. Bartholdi, W. L. C. Vaz, and T. M. Jovin, unpublished results). A cooperative thermal transition in the diffusion parameter, the rotational correlation time, is not observed, but there is evidence for a time-dependent aggregation below the lipid phase transition.

Acknowledgments

We thank Dr. H. H. Füldner for useful discussions.

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Independent Folding Regions in Aspartokinase–Homoserine Dehydrogenase†

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ABSTRACT: The folding of two monofunctional fragments of aspartokinase–homoserine dehydrogenase I has been studied. One of these fragments corresponds to the kinase activity and the N-terminal part of the polypeptide chain; the other one corresponds to the dehydrogenase activity and to the C-terminal part of the chain. Both fragments are able to refold into an enzymatically active conformation after complete disruption of their native structure. The kinase fragment folds up into an active monomeric species. The dehydrogenase fragment first folds up into an inactive monomeric species and then associates into an active dimeric species. These two fragments thus correspond to regions capable of autonomous folding. The folding of each of these fragments is compared to that of the corresponding region in the intact aspartokinase–homoserine dehydrogenase I reported previously [Garel, J. R., & Dautry-Varsat, A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3379–3383]. It is concluded that the N- and C-terminal regions of the intact polypeptide chain behave as independent folding units. A model of the sequence of steps involved in the folding process of aspartokinase–homoserine dehydrogenase I is presented; its relevance to the evolution of this protein is also discussed.

Two different approaches, X-ray crystallography and fragmentation studies, have shown that the tertiary structure of large polypeptide chains results from the assembly of several compact regions called domains.[1] Indeed, the structure of large proteins, as seen by X-ray crystallography, reveals the presence of compactly folded blocks, each corresponding sometimes to a continuous segment of the polypeptide chain (Liljas & Rossmann, 1974; Schulz & Schirmer, 1979). Also, it is often possible to obtain smaller folded fragments from large proteins by enzymatic or chemical cleavage of the polypeptide chain or by nonsense mutation in the corresponding gene. These fragments can be considered folded when they retain some of the properties of the protein they derive from: globular structure, ability to bind specific ligands, oligomeric state, recognition by specific antibodies, enzymatic activity, etc. (e.g., Porter, 1959; Setlow & Kornberg, 1972; Véron et al., 1972; Cohen & Holzer, 1979). Some of these properties even suggest that, when stable, this folded conformation resembles that of the corresponding segment of the chain in the folded intact protein. Besides, some of these fragments represent autonomous folding units, as judged by their capacity to resume this stable conformation after complete unfolding (Jacobson & Rosenbusch, 1976; Geisler & Weber, 1976; Högberg-Raibaud & Goldberg, 1977; Ghelis et al., 1978; Dautry-Varsat & Garel, 1978). The existence, stability, and ability to refold of these compact regions suggest that they play a role in the folding process of the entire protein. This is usually described by a sequential mechanism: the folding of a large polypeptide chain would begin with the independent folding of different segments into compact units, which would then assemble into a folded chain. In the case of an oligomeric protein, the final structure is achieved by the association between such folded chains.

We have previously investigated the folding process of a large protein, the bifunctional enzyme aspartokinase I–homoserine dehydrogenase I (AK-HDH)[2] from *Escherichia coli* K12. This enzyme is a tetramer of four identical subunits of molecular weight 89 000 (Falcoz-Kelly et al., 1972). Each chain carries the sites for both enzymatic activities, and its

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[1] We use the word region to design compactly folded parts of the polypeptide chain which have been defined functionally and keep the word domain to design such compactly folded parts defined structurally, by X-ray crystallography. Since the X-ray structure of AK-HDH is unknown, the correspondence between these two definitions cannot be made at the present time.

[2] Abbreviations used: AK-HDH, aspartokinase I–homoserine dehydrogenase I (EC 2.7.2.4 and EC 1.1.1.3); AK fragment, derivative of AK-HDH obtained by an ochre mutation of the corresponding gene; HDH fragment, derivative of AK-HDH obtained by limited proteolysis; Gdn-HCl, guanidine hydrochloride.

0006-2960/81/0420-1396$01.25/0