Cereghini, S., Herbomel, P., Jouanneau, J., Saragosti, S., Katinka, M., Bourachot, B., De Crombrugghe, B., & Yaniv, M. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 935-944.
Chao, M. V. (1980) Doctoral Dissertation, University of California, Los Angeles.
Chao, M. V., Gralla, J., & Martinson, H. G. (1979) *Biochemistry 18*, 1068-1074.
Drew, H. R., & Travers, A. A. (1985) *J. Mol. Biol. 186*, 773-790.
Hiwasa, T., Segawa, M., Yamaguchi, N., & Oda, K. (1981) *J. Biochem. (Tokyo) 89*, 1375-1389.
Jakobovits, E. B., Bratosin, S., & Aloni, Y. (1980) *Nature (London) 285*, 263-265.
Klevan, L., & Crothers, D. (1977) *Nucleic Acids Res. 4*, 4077-4089.
Kunkel, G. R., & Martinson, H. G. (1981) *Nucleic Acids Res. 24*, 6869-6888.
Lebowitz, P., & Weissman, S. M. (1979) *Curr. Top. Microbiol. Immunol. 87*, 43-172.
Linxweiler, W., & Hörz, W. (1984) *Nucleic Acids Res. 12*, 9395-9413.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787-3794.
Marini, J. C., Levene, S. D., Crothers, D. M., & Englund, P. T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7664-7668.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499-559.
McGhee, J. E., & Felsenfeld, G. (1980) *Annu. Rev. Biochem. 49*, 1115-1156.
Nedospasov, S. A., & Georgiev, G. P. (1980) *Biochem. Biophys. Res. Commun. 92*, 532-539.
Persico-DiLauro, M., Martin, R. G., & Livingston, D. M. (1977) *J. Virol. 24*, 451-460.
Rall, S. C., Okinaka, R. T., & Strniste, G. F. (1977) *Biochemistry 16*, 4940-4944.
Robinson, G. W., & Hallick, L. M. (1982) *J. Virol. 41*, 78-87.
Saragosti, S., Moyne, G., & Yaniv, M. (1980) *Cell (Cambridge, Mass.) 20*, 65-73.
Scott, W. A., & Wigmore, D. J. (1978) *Cell (Cambridge, Mass.) 15*, 1511-1518.
Simpson, R. T., & Stein, A. (1980) *FEBS Lett. 111*, 337-339.
Sogo, J. M., Stahl, H., Koller, T., & Knippers, R. (1986) *J. Mol. Biol. 189*, 189-204.
Tapper, D. P., & De Pamphilis, M. L. (1980) *Cell (Cambridge, Mass.) 22*, 97-108.
Tatchell, K., & Van Holde, K. E. (1979) *Biochemistry 18*, 2871-2880.
Tooze, J. (1981) *DNA Tumor Viruses: Molecular Biology of Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Trifonov, E. N., & Mengeritsky, G. (1984) *J. Virol. 52*, 1011-1012.
Varshavsky, A. J., Sundin, O. H., & Bohn, M. J. (1978) *Nucleic Acids Res. 5*, 3469-3477.
Waldeck, W., Föhring, B., Chowdhury, K., Gruss, P., & Sauer, G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5964-5968.
Wasylyk, B., Oudet, P., & Chambon, P. (1979) *Nucleic Acids Res. 7*, 705-713.
Weaver, D. T., Fields-Berry, S. C., & De Pamphilis, M. L. (1985) *Cell (Cambridge, Mass.) 41*, 565-575.
Zannis-Hadjopoulos, M., Chepelinsky, A. B., & Martin, R. G. (1983) *J. Mol. Biol. 165*, 599-607.

Competitive Labeling as an Approach to Defining the Binding Surfaces of Proteins: Binding of Monomeric Insulin to Lipid Bilayers†

Raymond P. Oomen and Harvey Kaplan*
Department of Biochemistry, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada
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ABSTRACT: The free monomeric form of insulin is known to adsorb strongly to many different surfaces. A question of physiological relevance for which no previous studies have been reported is whether the monomeric form of insulin binds to lipid bilayers. In order to answer this question, it is necessary to carry out studies at the very dilute concentrations (less than 10^{-6} M) necessary to obtain this species. We have approached this problem by applying the method of competitive labeling [Hefford, M. A., Evans, R. M., Oda, G., & Kaplan, H. (1985) *Biochemistry 24*, 867-874] to study insulin at concentrations as low as 3×10^{-8} M, in the presence and absence of large unilammelar liposomes. With 1-fluoro-2,4-dinitrobenzene as the labeling reagent, the relative chemical reactivities of the functional groups of insulin were found to decrease markedly when insulin was incubated with liposomes consisting of egg lecithin and cholesterol (2:1 mol/mol) in 1.0 M KCl, pH 7.5 at 37 °C. The decrease for each functional group was found to directly correlate with its proximity to the dimer-forming surface of the monomer. It is concluded that insulin binds to lipid bilayers in a specific orientation, with the dimer-forming surface interacting with the bilayer. These results demonstrate the feasibility of applying competitive labeling to obtain structure–function relationships of membrane-interactive proteins in general and monomeric insulin in particular.

Insulin, at the very dilute physiological concentrations where the free monomer is the predominant species, is widely known to strongly adsorb[1] to many different surfaces; in spite of this, there have been very few quantitative studies on this phenomenon. Hollenberg and Cuatrecasas (1976) reported an

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[1] Adsorption refers to the association between two components where the orientation is random or not defined. Binding refers to an association between two components where the orientation is known to be nonrandom.

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adsorption to glass containers that showed some of the same properties as specific binding to receptors. Concentration-dependent adsorption to glass surfaces was confirmed by Kaplan et al. (1984), who also reported strong adsorption to Teflon and polystyrene surfaces. In view of the fact that monomeric insulin adsorbs to such a variety of different surfaces, the question arises as to whether this property of insulin is of any physiological relevance. In the circulatory system in which monomeric insulin is transported, the most prevalent surfaces are the cell membranes of various tissues. The objective of this study was to determine the extent and nature of the interaction of monomeric insulin with lipid bilayers.

There have been no reports on whether the free insulin monomer associates with lipid bilayers. However, Wiessner and Hwang (1982) reported that insulin dimers adsorb to small, highly curved unilamellar vesicles at temperatures below 37 °C but not to large unilamellar vesicles. Schwinke et al. (1983) found that insulin monomers alter the surface tension of lipid monolayers and concluded that monomers can penetrate the lipid–water interface.

The dearth of information may be related to the lack of sensitive physical methods for studying proteins in very dilute solution and to the fact that insulin monomers adsorb strongly to the surfaces of the containing vessels, complicating the interpretation of results. It has been demonstrated (Kaplan et al., 1984; Hefford et al., 1986) that the insulin monomer in 1.0 M salt does not adsorb to glass surfaces, and this finding provides a means for eliminating extraneous adsorption. In order to achieve the sensitivity necessary to study the properties of proteins in very dilute solution, we have applied the modified method of competitive labeling described by Hefford et al. (1985). This approach measures the relative chemical reactivities of nucleophilic functional groups in proteins [for reviews see Glazer (1976) and Bosshard (1979)]. Reactivities reflect the pK and microenvironment of the functional groups and may be combined with sequence and X-ray crystallographic data to arrive at structure–function relationships. If insulin binds or adsorbs to unilamellar liposomes, this should alter the chemical properties of the functional groups in a manner related to the orientation of the molecule on the surface. In this paper we carry out studies in the presence and absence of liposomes to an insulin concentration of 3×10^{-8} M, where the monomer is the predominant species, present at approximately 98 mol % (DeMeyts et al., 1978. Competitive labeling was done in the absence and presence of large unilammelar liposomes, in order to determine whether and how monomeric insulin interacts with a lipid bilayer.

Experimental Procedures

Materials. Bovine pancreatic insulin was obtained from Sigma Chemical Co. (St. Louis, MO). The purity of this preparation was verified by C18 reversed-phase HPLC.[2] Egg phosphatidylcholine (PC; type V-E), N_2ph-F, alanylalanine, N^{α}-acetylhistidine, and dinitrophenyl derivatives of phenylalanine, glycine, tyrosine, and lysine were also obtained from Sigma. Amersham Corp. (Oakville, Ontario, Canada) supplied [^{14}C]N_2ph-F and [^{3}H]N_2ph-F and NEN Canada (Lachine, P.Q., Canada) the Aquasol-2 for scintillation counting.

Sample Preparation. A stock solution (25.0 mL) containing an equimolar (1×10^{-4} M) mixture of glycine, alanylalanine, and insulin was prepared in a buffer of 1.0 M KCl and 5 mM Na_2HPO_4 at pH 7.5. A 50-fold dilution of a portion (0.5 mL) of the stock solution was made, and aliquots of this working solution (2×10^{-6} M) were transferred to screw-capped Pyrex test tubes, to give the desired concentrations. The remainder of the stock solution was lyophilized in preparation for ^{14}C labeling.

A stock liposome preparation was prepared following the reverse-phase evaporation method of Szoka and Papahadjopoulos (1978). A mixture of egg PC (120 μmol) and cholesterol (60 μmol) was dried down under nitrogen and redissolved in 4.5 mL of peroxide-free diethyl ether. Buffer was added (1.5 mL), and the two-phase system was sonicated with a Branson sonicator using a needle tip probe set at maximum allowable power in spaced bursts of 1-min duration. The temperature of the preparation during sonication was maintained at 22 °C with a water bath, and the organic solvent was removed on a Buchi rotavapor at 37–40 °C under aspirator-reduced pressure. This was continued for 5 min past the point at which ether could no longer be detected by odor. The preparation was diluted with 1.5 mL of buffer, evaporated (30 min), and set aside as a stock liposome solution with a total lipid concentration of 6 mM.

Competitive Labeling. The competitive labeling method used by Hefford et al. (1985) was employed in this study with minor modifications.

(*A*) *^{3}H Trace Labeling.* For liposome samples, 0.5-mL aliquots of the liposome stock solution were added to aliquots of insulin stock solution designed to give four final insulin and internal standard concentrations of 10^{-6}, 3×10^{-7}, 10^{-7}, and 3×10^{-8} M when made up to a final volume of 2.0 mL. Buffer was added to reach this volume, and the pH was adjusted if necessary with 1.0 N NaOH or 1.0 N HCl to pH 7.5. For the control samples, buffer was substituted for the liposome preparation. Mixtures were incubated for 3 h in a 37 °C thermostated water bath with gentle agitation. An aliquot (10 μL) of acetonitrile containing [^{3}H]N_2ph-F (18.1 μCi, specific radioactivity 16.6 Ci/mmol) was added to each sample with vigorous stirring and the reaction left to proceed in the dark for 18 h. Complete derivatization of all groups was accomplished by making each sample approximately 8 M in urea (through addition of 2.0 g of urea), adding 0.25 g of $NaHCO_3$ and 50 μL of 50% N_2ph-F in acetonitrile (v/v), and reacting for 18 h at room temperature in the dark. All labelings were carried out in triplicate.

(*B*) *Preparation and Addition of [^{14}C]Insulin and Internal Standards.* The lyophilized stock solution was dissolved in a minimum volume of 8 M urea, saturated with $NaHCO_3$. To this was added 200 μL of a 25% [^{14}C]N_2ph-F solution in acetonitrile (v/v) (2.0 mmol, 125 μCi/mmol), and the reaction was allowed to proceed overnight. The ^{14}C-labeled insulin and internal standard solution was split into equal aliquots, one being added to each ^{3}H trace labeled sample.

(*C*) *Internal Standard Isolation and Purification.* Carrier was added to each sample in the form of dinitrophenylated bovine serum albumin (5 mg), and N_2ph derivatives of glycine and Ala-Ala (0.5 mg of each per sample). The samples were then extracted with three 5-mL aliquots of $CHCl_3$ to remove the bulk of the lipid and then brought to pH 2 with concentrated HCl. Further extractions with four 5-mL aliquots of diethyl ether were carried out, and the extracts, containing the dinitrophenylated internal standards were dried. The internal standards were then dissolved in 95% ethanol, spotted on TLC silica plates, and separated with a $CHCl_3$–benzyl alcohol–glacial acetic acid (70:30:3 v/v) solvent system, ac-

[2] Abbreviations: Ala-Ala, alanylalanine; HPLC, high-performance liquid chromatography; L/C, liposome sample reactivity/control sample reactivity; N_2ph-F, 1-fluoro-2,4-dinitrobenzene; N_2ph, 2,4-dinitrophenyl.

cording to Brenner et al. (1961). Alternatively, purification of the internal standards was accomplished with a 5-μm reverse-phase C18 silica column (Beckman Ultrasphere-ODS, 0.46 × 25 cm), with a 10-μm C18 precolumn (3.2 × 40 mm), at 22 °C. Eluate was analyzed at 320 nm with an Hitachi 100-40 variable-wavelength spectrophotometer. The solvent system employed various gradients of HPLC-grade acetonitrile against an ammonium formate buffer, 35 mM, pH 3.0 (Cockle et al., 1982), at a flow rate of 1 mL/min.

Functional Group Isolation and Purification. The aqueous phase was centrifuged on a bench-top centrifuge, and the pellet was washed with successive 5-mL aliquots of distilled H_2O, 50% acetone (v/v in distilled H_2O, twice), and 100% acetone. To the dried pellet was added 1 mL of 6 M HCl; the tubes were sealed under vacuum and subjected to hydrolysis for 18 h at 100 °C. The hydrolysate was extracted with four 2-mL aliquots of diethyl ether and dried; the N^{α}-[^{3}H/^{14}C]N_2ph-glycine and N^{α}-[^{3}H/^{14}C]N_2ph-phenylalanine were separated on silica plates by two-dimensional TLC with toluene–pyridine–2-chloroethanol–0.8 N ammonia (100:30:60:60 v/v) for the first dimension and benzene–pyridine–glacial acetic acid (80:20:2 v/v) for the second dimension (Brenner et al., 1961). Alternatively, the N_2ph-labeled N-termini were separated by HPLC as above.

The aqueous phase from the hydrolysate after ether extraction was subjected to high-voltage paper electrophoresis at pH 2.1 (3500 V, 45 min) to separate $N^{\pi/\tau}$-[^{3}H/^{14}C]N_2ph-histidine, N^{ϵ}-[^{3}H/^{14}C]N_2ph-lysine, and O-[^{3}H/^{14}C]N_2ph-tyrosine. The bands were removed and subjected to a second electrophoresis (pH 2.1, 3500 V, 75 min) in order to completely remove trace contamination.

Liquid Scintillation Counting. All samples were dried in borosilicate counting vials and dissolved in 100 μL of 0.01 M HCl, and 10 mL of Aquasol-2 was added. Scintillation counting was carried out on a programmable Beckman 1800 scintillation counter equipped with automatic quench correction and dpm converter.

Results and Discussion

The rationale behind this study is that the binding of a protein molecule to a surface will alter the chemical reactivity of the functional groups in a characteristic way depending on the orientation of the molecule on the surface. If a group is directly involved in binding, its reactivity should decrease markedly. On the other hand, groups on the opposite surface of the molecule would still be exposed to solvent and should retain their usual reactivity. Between these two extremes, it is expected that groups will encounter differing degrees of steric hindrance, and the decrease in reactivity will be directly related to their proximity to the binding surface.

Internal Standards. Since the measured volume of tritiated label added in each reaction is very small (10 μL), it is difficult to achieve precise reproducibility among samples. In the competitive labeling procedure, this problem is corrected by including an internal standard which at any given time during the reaction will have the same amount of label available as the functional groups under study. The ideal internal standard is one that does not interact with the other components of the system, viz., liposomes and insulin.

According to theory (Kaplan et al., 1971), the specific radioactivity of the internal standards glycine and Ala-Ala should be identical over the entire concentration range studied if the same amount of label was added in each experiment. As mentioned above, this is very difficult to achieve; however, the constancy of the ^{3}H/^{14}C ratios will give some indication as to whether the standards are interacting with the liposomes.

Table I: ^{3}H/^{14}C Ratios of Internal Standards[a]

internal standard	concn (M)	liposome	control
Ala-Ala	1×10^{-6}	4.23 ± 0.04	4.20 ± 0.21
	3×10^{-7}	4.18 ± 0.91	3.91 ± 0.10
	1×10^{-7}	4.52 ± 0.42	6.48 ± 1.78
	3×10^{-8}	3.75 ± 1.43	4.38 ± 0.68
glycine	1×10^{-6}	0.70 ± 0.01	0.84 ± 0.02
	3×10^{-7}	0.54 ± 0.07	0.62 ± 0.07
	1×10^{-7}	0.40 ± 0.14	0.68 ± 0.24
	3×10^{-8}	0.26 ± 0.12	0.53 ± 0.09

[a] Average of three determinations with standard deviation.

Table II: Functional Group ^{3}H/^{14}C Ratios[a]

group	concn (M)	control (C)	liposome (L)	L/C[b]
Al Gly	1×10^{-6}	1.999 ± 0.052	0.667 ± 0.059	0.56
	3×10^{-7}	0.681 ± 0.022	0.133 ± 0.004	0.19
	1×10^{-7}	0.510 ± 0.080	0.054 ± 0.002	0.11
	3×10^{-8}	0.153 ± 0.001	0.023 ± 0.003	0.15
B1 Phe	1×10^{-6}	0.613 ± 0.038	0.618 ± 0.005	1.01
	3×10^{-7}	0.117 ± 0.010	0.164 ± 0.009	1.40
	1×10^{-7}	0.066 ± 0.012	0.069 ± 0.005	1.05
	3×10^{-8}	0.042 ± 0.001	0.112 ± 0.003	2.68
B29 Lys	1×10^{-6}	0.227 ± 0.016	0.480 ± 0.089	2.11
	3×10^{-7}	0.218 ± 0.004	0.78 ± 0.014	0.36
	1×10^{-7}	0.128 ± 0.013	0.055 ± 0.013	0.43
	3×10^{-8}	0.228 ± 0.013	0.063 ± 0.003	0.28
His (av of 2)	1×10^{-6}	0.378 ± 0.012	0.148 ± 0.001	0.39
	3×10^{-7}	0.168 ± 0.001	0.037 ± 0.002	0.22
	1×10^{-7}	0.076 ± 0.011	0.016 ± 0.001	0.21
	3×10^{-8}	0.047 ± 0.007	0.021 ± 0.001	0.45
Tyr (av of 4)	1×10^{-6}	0.244 ± 0.008	0.392 ± 0.009	1.61
	3×10^{-7}	0.161 ± 0.005	0.074 ± 0.004	0.46
	1×10^{-7}	0.088 ± 0.002	0.055 ± 0.002	0.63
	3×10^{-8}	0.075 ± 0.001	0.032 ± 0.004	0.42

[a] Experiments were done in triplicate and normalized with respect to Ala-Ala. [b] See text for definition.

Table I shows that the reactivity of Ala-Ala does not change substantially whether liposomes are present or not. This establishes that the kinetics of the reaction follow a first-order rate law, as required for competitive labeling, and that Ala-Ala does not interact with the liposomes.

Glycine that is free in solution has an approximate 5-fold lower reactivity than Ala-Ala due primarily to its higher pK value for the α-amino group. Reactivity is also decreased at lower concentrations of the nucleophile. When liposomes are present, there is a 15–20% decrease in reactivity at the higher concentrations and a 50% decrease at 3×10^{-8} M. Since the decrease is concentration-dependent, adsorption is most likely taking place at the low concentrations.

The effect of liposomes on the chemical properties of insulin at each concentration is quantified by normalizing all reactivities with respect to the reactivity of the internal standards (glycine and Ala-Ala). This must be done at each concentration for both the control (C) sample and the liposome-containing (L) samples. Table II shows the data obtained with the standard deviations. The L/C ratio then becomes an expression that describes the altered reactivity of a particular group with reference to a standard. If L/C is greater than unity, the presence of liposomes results in an increase of chemical reactivity; if L/C is less than unity, the reactivity is diminished. Figure 1 shows the L/C ratios for all of the insulin functional groups tested, relative to the internal standards glycine and Ala-Ala. The tyrosine and histidine ratios represent the average reactivities of the four tyrosine and two histidine residues of bovine insulin. Figure 1 gives the L/C reactivity profile as a function of insulin concentration with Ala-Ala (Figure 1A) and glycine (Figure 1B) as internal standards. In each case, the reactivity of all groups except

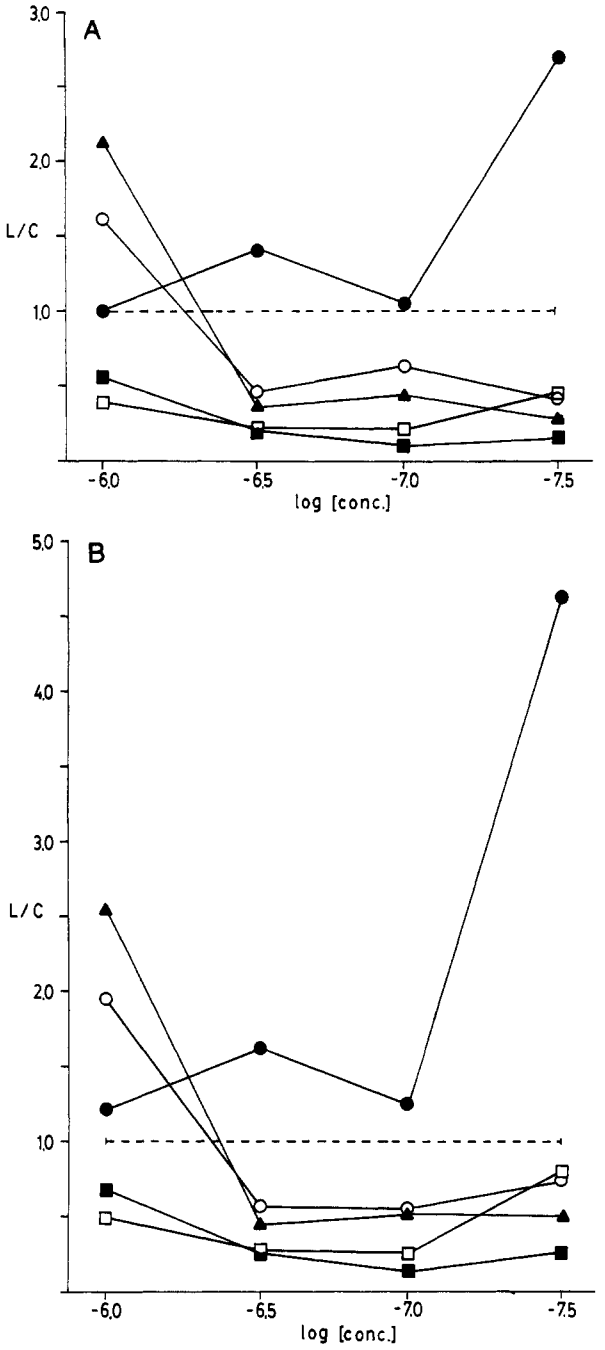

FIGURE 1: Reactivity L/C–concentration profiles of functional groups of insulin with respect to internal standards alanylalanine (A) and glycine (B): (●) phenylalanine; (○) tyrosines; (□) histidines; (■) glycine; (▲) lysine.

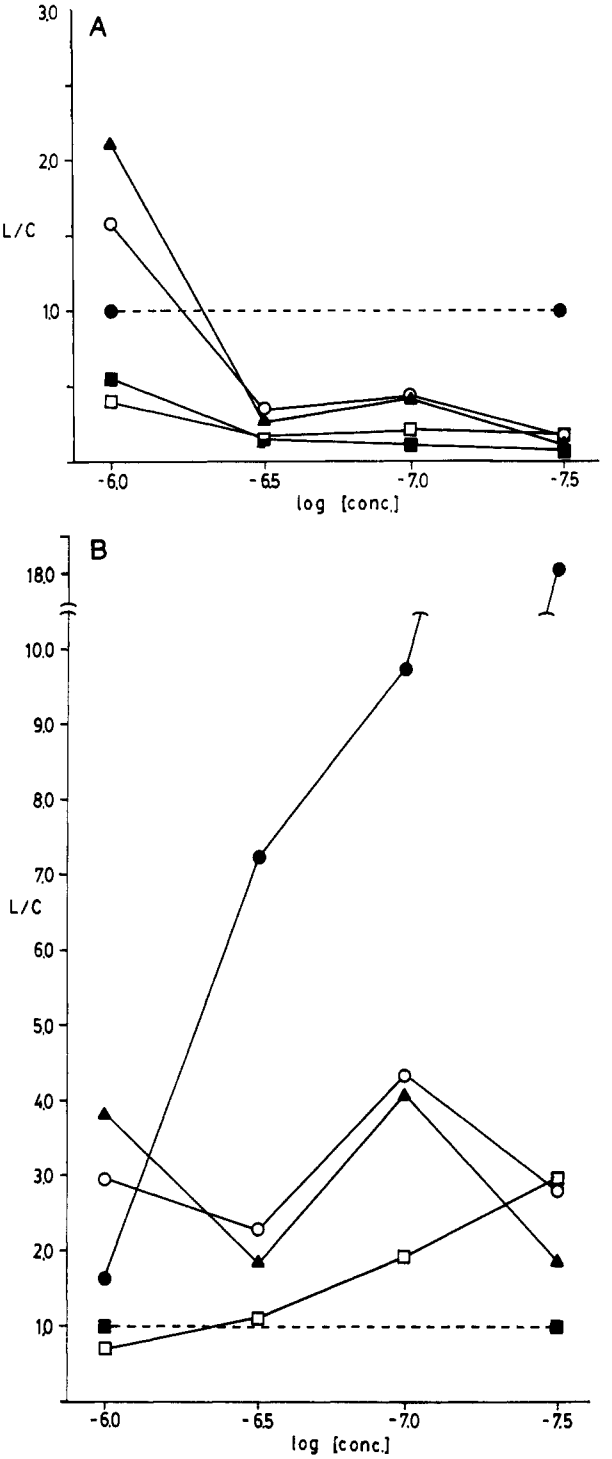

FIGURE 2: Reactivity L/C–concentration profiles of functional groups of insulin with respect to B1 Phe (A) and A1 Gly (B) N-termini as internal standards: (●) phenylalanine; (○) tyrosines; (□) histidines; (■) glycine; (▲) lysine.

that of the phenylalanine amino terminus decreases as the concentration of insulin is decreased below 10^{-6} M where the free monomer predominates. The A1 glycine amino-terminus shows the largest reduction in reactivity to approximately 20% of that in the absence of liposomes. It should be noted that for most of the data points, the L/C ratios calculated with respect to free glycine in solution are 15–20% greater than the corresponding values calculated with respect to Ala-Ala as the internal standard. We feel that this is further evidence that free glycine adsorbs to the liposomes. If, as has been mentioned previously, free glycine is increasingly adsorbed at the lower concentrations, this would account for the apparent increases in functional group reactivity observed at 3×10^{-8} M in Figure 1B. The dipeptide Ala-Ala does not appear to interact with liposomes under the experimental conditions employed and is therefore more suitable for use as an internal standard than glycine.

Functional Groups. The use of noninteracting internal standards is a necessary step in establishing that adsorption of insulin takes place but does not answer the question as to whether the molecule is binding in a specific orientation or associating in a nonspecific manner. Another approach is to treat each of the functional groups in the molecule as an internal standard. If this is done in turn with each functional group, and depending on the location and number of groups, it should be possible to obtain a quantitative assessment of the

chemical topology of the bound molecule. Figure 2 shows the L/C profile of the functional groups after normalization with respect to B1 Phe and A1 Gly. The most striking feature of the B1 Phe L/C profile (Figure 2A) is the resemblance to the profiles obtained with the internal standards as the reference nucleophiles. Quantitatively, it appears that access of the labeling reagent to the B-chain N-terminal α-amino group, in the free and bound states, is as unrestricted as the access to the internal standard nucleophiles. The relative decrease in reactivity of the other functional groups immediately suggests that the B1 Phe has a similar environment in the free and bound states of insulin whereas the other groups are sterically hindered in the bound state.

Examination of the 2Zn crystal structure shows that B1 Phe is located at the opposite end of the molecule from the dimer-forming surface in both molecules 1 and 2 [the nomenclature of the Beijing Insulin Group is used, see Chothia et al. (1983)]. An earlier suggestion that upon dissociation of the dimer residues B1–B3 may pack more closely to the surface of the molecule (Blundell, 1972) is based on the 2Zn crystal structure, in which B1 Phe is in a surface pocket bordering the dimer–dimer contact region of the insulin hexamer. A large body of evidence, including atomic temperature factor analysis (Tainer et al., 1985), antigenicity studies of exposed surface areas (Schroer et al., 1983), and earlier labeling and modification studies (Blundell et al., 1972), supports the idea that in aqueous media the B1 Phe α-amino group extends into solution and is probably not tightly packed against the insulin molecule or even loosely constrained by molecular surface orbitals. Therefore, the α-amino group of B1 Phe is an excellent vantage point from which to gauge the change in chemical reactivities that the other functional groups of the insulin molecule undergo on binding of insulin.

If the A-chain glycine amino terminus is taken as the reference nucleophile, the L/C profile in Figure 2B is obtained. Relative to A1 Gly, the B-chain N-terminus appears to have a greatly augmented reactivity at all concentrations, increasing to an 18-fold greater reactivity at the lowest concentration. This apparent increase may reflect a continuing decrease in A1 Gly reactivity or, conversely, may reflect a real increase attributable in part to some membrane-associated conformational change that increases B1 Phe reactivity. All the other functional groups also show an enhanced reactivity over that of glycine.

Like B1 Phe, A1 Gly is also accessible to the solvent in the monomer, being situated in a "compact, surface pocket" (Blundell et al., 1972). Although the position of A1 Gly is altered by the conformational changes represented in the 2Zn and 4Zn crystal structures (Chothia et al., 1983), it is constrained by its function as a shielding group for the hydrophobic core of the insulin molecule. The A1 Gly amino group is also implicated in receptor binding (Pullen et al., 1976; Saunders, 1981; Gammeltoft, 1984) and is adjacent to the hydrophobic dimer-forming surface of the molecule. It is not unreasonable to expect that if the insulin monomer binds to the liposome by apposition of its hydrophobic surface, the reactivity of A1 Gly will be diminished. However, flexibility and conformation of the molecule at positions B29–B30 has to be critically assessed since these residues represent a topological projection separating the dimer-forming face from the vicinity of A1 Gly in the 2Zn dimer crystal model. Conceptually, therefore, it may be useful to consider A1 Gly and B1 Phe as lying on opposite ends of a spectrum of solvent and electrophile accessibility when the protein is bound to the liposome surface, and that as B1 Phe may be a sensitive indicator of conditions external to the bulk molecule (in the monomer form), so A1 Gly may be a suitable reporter group for a defined region of the molecular surface that takes part in the binding process. Figure 3 illustrates our conclusions regarding the orientation of these two groups and the other functional groups of the insulin monomer bound to the liposome.

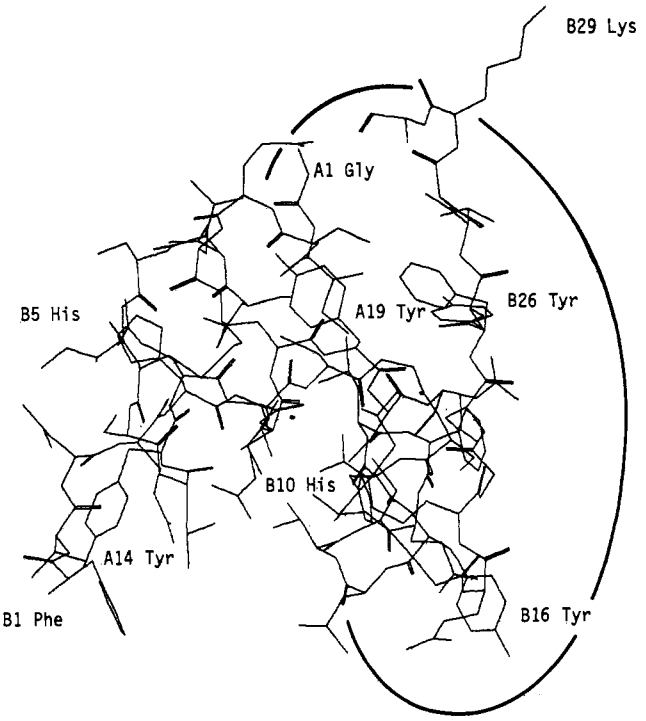

FIGURE 3: Proposed binding region of insulin monomer. The plane of the dimer-forming surface lies perpendicular to the plane of the paper and runs vertically on the far right of the molecule. The diagram was derived from the X-ray crystallographic structure of the monomeric unit in the insulin hexamer (Dodson et al., 1979). Coordinates were obtained from the Brookhaven Protein Data Bank and displayed on an Evans and Sutherland PS 300 color graphics workstation.

The alterations in the reactivities of the lysine, tyrosine, and histidine functional groups are intermediate between those of the phenylalanine and glycine amino termini. In the case of the α-amino group of B29 Lys, the X-ray data show that, while it is close to the dimer-forming surface, it is one of the most flexible parts of the molecule [Tainer et al. (1985) and references cited therein]. Its decrease in reactivity is therefore consistent with the dimer-forming surface being the site at which insulin binds to liposomes. For the other functional groups it is not possible to draw any conclusions since more than one group is involved. However, on the basis of our proposed binding site, it is possible to predict that the two tyrosines of the B chain would show markedly decreased reactivity while those on the A chain would not be expected to have a decreased reactivity. For the two histidine residues we would predict a larger decrease for the B10 histidine than for the B5 histidine (Figure 3).

At 10^{-6} M, the tyrosine and lysine residues show an enhanced reactivity in the presence of liposomes. At present we cannot offer an explanation for this observation except to state that it is highly reproducible. Similarly, we cannot completely account for the apparently enhanced L/C ratio of the B1 Phe N-terminus at 3×10^{-8} M. It is quite possible that these cases reflect conformational changes from dimer association with liposomes in the former case and from monomer–liposome associations in the latter. These effects, whatever their origin, are relatively minor in the context of the overall pattern of the

reactivity changes, which clearly show that insulin binds to lipid bilayers in a highly specific manner involving its dimer-forming surface.

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REFERENCES

Blundell, T., Dodson, G. G., Hodgkin, D. C., & Mercola, D. A. (1972) *Adv. Protein Chem. 26*, 279-402.
Bosshard, H. R. (1979) *Methods Biochem. Anal. 25*, 273-301.
Brenner, M., Niederwieser, A., & Pataki, G. (1961) *Experientia 17*, 145.
Chothia, C., Lesk, A. M., Dodson, G. G., & Hodgkin, D. C. (1983) *Nature (London) 302*, 500-505.
Cockle, S. A., Kaplan, H., Hefford, M. A., & Young, M. N. (1982) *Anal. Biochem. 125*, 210-216.
DeMeyts, P., Van Obberghen, E., Roth, J., Wollmer, A., & Brandenburg, D. (1978) *Nature (London) 273*, 504-509.
Dodson, E. J., Dodson, G. G., Hodgkin, D. C., & Reynolds, C. D. (1979) *Can. J. Biochem. 57*, 469-479.
Gammeltoft, S. (1984) *Physiol. Rev. 64*, 1321-1378.
Glazer, A. N. (1976) in *The Proteins*, 3rd ed. (Neurath, H., & Hill, R. L., Eds.) pp 1-103, Academic, New York.
Hefford, M. A., Evans, R. M., Oda, G., & Kaplan, H. (1985) *Biochemistry 24*, 867-874.
Hefford, M. A., Oda, G., & Kaplan, H. (1986) *Biochem. J. 237*, 663-668.
Hollenburg, M. D., & Cuatrecasas, P. (1976) *Methods Mol. Biol. 9*, 429-477.
Kaplan, H., Stevenson, K. J., & Hartley, B. S. (1971) *Biochem. J. 124*, 289-299.
Kaplan, H., Hefford, M. A., Chan, A. M.-L., & Oda, G. (1984) *Biochem. J. 217*, 135-143.
Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J., & Gammeltoft, S. (1976) *Nature (London) 259*, 369-373.
Saunders, D. J. (1981) *Biosci. Rep. 1*, 485-495.
Schroer, J. A., Bender, T., Feldmann, R. J., & Kim, K. J. (1983) *Eur. J. Immunol. 13*, 693-700.
Schwinke, D. L., Ganesan, M. G., & Weiner, N. D. (1983) *J. Pharm. Sci. 72*, 244-248.
Szoka, F., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194-4198.
Tainer, J. A., Getzoff, E. D., Paterson, Y., Olsen, A. J., & Lerner, R. A. (1985) *Annu. Rev. Immunol. 3*, 501-535.
Wiessner, J. H., & Hwang, K. J. (1982) *Biochim. Biophys. Acta 689*, 490-498.

Inhibition of Protein Cross-Linking in Ca^{2+}-Enriched Human Erythrocytes and Activated Platelets†

L. Lorand,* N. Barnes, J. A. Bruner-Lorand, M. Hawkins, and M. Michalska

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60201

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ABSTRACT: Treatment of human erythrocytes with Ca^{2+}, in the presence of ionophore A23187, causes the formation of high molecular weight ($>10^6$) membrane protein polymers. This phenomenon, known to involve cross-linking of essentially all of the band 4.1 and 2.1 (ankyrin) proteins, as well as some spectrin, band 3, and hemoglobin molecules, could be prevented by preincubating the cells with a noncompetitive inhibitor of intrinsic transglutaminase, 2-[3-(diallylamino)propionyl]benzothiophene, at concentrations of about (3-6) $\times 10^{-4}$ M. The compound also eliminated the proteolytic breakdown of the two major transmembrane proteins band 3 and glycophorin, which would otherwise occur during the Ca^{2+} loading of fresh human red cells. In addition, the inhibitor effectively blocked the formation of a cross-linked protein polymer in thrombin-activated human platelets.

Elevation of the intracellular concentration of Ca^{2+} ions leads to marked changes in the covalent structures of proteins in human erythrocytes, as is evident from the examination of the membranes of Ca^{2+}-treated cells. In general, such protein modifications fall into two categories: polymerization and proteolytic degradation. Polymerization might occur (i) through reaction of proteins with bifunctional cross-linking agents, e.g., malonedialdehyde, generated from lipid peroxidation (Hochstein & Jain, 1981; Allen et al., 1984), (ii) through production of disulfides (Liu et al., 1977; Kosower et al., 1981), or (iii) through N^{ϵ}-(γ-glutamyl)lysyl side-chain peptide bridges (Lorand et al., 1976, 1978, 1979b, 1980; Siefring et al., 1978; Bjerrum et al., 1981). The latter reaction of protein fusion is due to the activation of latent transglutaminase, and in the present work, we explore the possibility of preventing this type of protein polymerization in Ca^{2+}-loaded human red cells with a noncompetitive inhibitor of transglutaminase: 2-[3-(diallylamino)propionyl]benzo-

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