Vaz, W. L. C., Criado, M., Madeira, V. M. C., Schoellmann, G., & Jovin, T. M. (1982) *Biochemistry 21*, 5608-5612.
Walder, J. A., Chatterjee, R., Steck, T. L., Low, P. S., Musso, G. F., Kaiser, E. T., Rogers, P. H., & Arnone, A. (1984) *J. Biol. Chem. 259*, 10238-10246.
Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005-8018.
Waseem, A., & Steck, T. L. (1989) *Methods Enzymol. 173*, 513-519.
Yoshida, T. M., Jovin, T. M., & Barisas, B. G. (1989) *Rev. Sci. Instrum. 60*, 2924-2928.
Yu, J., & Steck, T. L. (1975) *J. Biol. Chem. 250*, 9176-9184.
Zachariasse, K., Vaz, W. L. C., Sotomayor, C., & Kuehnle, W. (1982) *Biochim. Biophys. Acta 688*, 323-332.

Rotational Diffusion of Band 3 in Erythrocyte Membranes. 2. Binding of Cytoplasmic Enzymes

Edmund D. Matayoshi,*,‡ William H. Sawyer,§ and Thomas M. Jovin*
Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Postfach 2841, W-3400 Göttingen, FRG
Received August 29, 1990; Revised Manuscript Received December 19, 1990

ABSTRACT: Time-resolved phosphorescence anisotropy has been used to study the rotational diffusion of eosin-labeled human erythrocyte band 3 in the presence of an enzyme bound at its cytoplasmic pole. With increasing amounts of G3PD (glyceraldehyde-3-phosphate dehydrogenase) added to ghosts, the infinite time anisotropy (r_∞) increases, and at saturating concentrations, very little decay of the anisotropy $r(t)$ occurs at all. These phenomena are reversed by elution of the enzyme with 150 mM NaCl. Prior proteolytic removal of the N-terminal 41-kDa cytoplasmic fragment of band 3 also disenables the G3PD effect. When ghosts are stripped of their residually bound G3PD, a small reduction in the fraction of immobile band 3 is observed. Finally, titration of band 3 sites with aldolase shows effects on the $r(t)$ qualitatively similar to those observed with G3PD. On the basis of our interpretation of the heterogeneous anisotropy decay of eosin-labeled band 3 [Matayoshi, E. D., & Jovin, T. M. (1991) *Biochemistry* (preceding paper in this issue)], we conclude that the binding of G3PD and aldolase results in the immobilization of band 3 oligomers.

In the preceding paper (Matayoshi & Jovin, 1991), we compared in detail the rotational diffusion of eosin-maleimide-labeled band 3 in human erythrocyte ghosts and intact cells. A question of particular interest which arises from these studies is whether the rotational mobility of band 3 in the intact cell is affected by the binding of cytoplasmic proteins. The N-terminal 41-kDa region of band 3, which is thought to protrude as an elongated structure into the cytoplasmic space (Low, 1986), contains binding sites for the three glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (G3PD)[1] (Yu & Steck, 1975; Tsai et al., 1982), aldolase (Strapazon & Steck, 1976, 1977; Murthy et al., 1981), and phosphofructokinase (Higashi et al., 1979; Jenkins et al., 1985), as well as hemoglobin (Shaklai et al., 1977; Salhany et al., 1980; Sayare & Fikiet, 1981; Casoly, 1983; Murthy et al., 1984; Walder et al., 1984; Chetrite & Casoly, 1985; Low, 1986; Premachandra, 1986). In addition, the catalytic activities of G3PD, aldolase, and phosphofructokinase are altered upon complexation with band 3 amino-terminal fragments. The interaction of these cytoplasmic proteins with band 3 is significant in vitro only under conditions of low ionic strength, and it might therefore be questioned whether such binding has any relevance to the erythrocyte (Maretzki et al., 1989; Masters, 1989). Indeed, in two studies, it was concluded that G3PD in the intact cell is not membrane-bound (Brindle et al., 1982; Rich et al., 1985). On the other hand, support for the existence of membrane-bound glycolytic enymes in the intact cell was obtained in other studies (Kliman & Steck, 1980; Solti et al., 1981; Fossel & Solomon, 1981; Jenkins et al., 1984). Steck and co-workers estimate that perhaps half to two-thirds of the total cellular content of G3PD, aldolase, and phosphofructokinase are bound to band 3 in the intact cell (Kliman & Steck, 1980; Jenkins et al., 1984). Such occupancy would require 15-20% of the 1 million band 3 monomers per cell. If one further assumes that about half of the band 3 is complexed with hemoglobin (Chetrite & Casoly, 1985), there would be sufficient band 3 to bind both hemoglobin and the glycolytic enzymes, as well as other membrane proteins such as bands 2.1, 4.1, and 4.2. Although the question as to whether cytoplasmic proteins are bound to band 3 in vivo must be considered still unresolved, it is evident that such interactions could be advantageous for coordinating cell flexibility and metabolism with transport functions, as has been noted by many authors [e.g., see Salhany and Gaines (1981), Gillies (1982), and Low (1986)].

In this paper, we report that the binding of G3PD and aldolase to band 3 in ghosts inhibits its rotational diffusion. A preliminary account of this work has appeared previously (Matayoshi et al., 1983).

Materials and Methods

Materials. Rabbit muscle G3PD and rabbit muscle aldolase were obtained from Sigma. All other materials were as described in Matayoshi and Jovin (1991).

Sample Treatments. Residually bound G3PD was removed from ghosts by incubation of ghosts for 20 min at room temperature either in 10 mM sodium phosphate/0.4 M NaCl, pH

* Correspondence should be addressed to either author.
‡ Present address: Abbott Laboratories, Department of Computer-Assisted Molecular Design, D-47E, Abbott Park, IL 60064.
§ Permanent address: Department of Biochemistry, University of Melbourne, Melbourne, Victoria 3052, Australia.

[1] Abbreviations: G3PD, glyceraldehyde-3-phosphate dehydrogenase; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonate.

0006-2960/91/0430-3538$02.50/0

Table I: Phosphorescence Anisotropy Decay of Eosin-Labeled Band 3[a]

	control ghosts		ghosts + 3 μM G3PD		G3PD-depleted ghosts[b]	
component, i	ϕ_i (μs)	r_{0i}	ϕ_i (μs)	r_{0i}	ϕ_i (μs)	r_{0i}
1	28	0.026	22	0.020	26	0.027
2	154	0.033	140	0.018	250	0.039
3	2060	0.040	5450	0.028	2770	0.044
	r_∞ = 0.068		r_∞ = 0.098		r_∞ = 0.053	

[a] Analyses of multiple time per channel records, as discussed in Matayoshi and Jovin (1991). The 5 μs per channel data are shown in Figure 1A. The ghosts in the above three samples were 24–36-h old; all measurements were at 37 °C, in 10 mM sodium phosphate, pH 7.4. [b] Ghosts were stripped of residual G3PD by incubation in 0.4 M NaCl for 20 min at 23 °C.

7.4 (McDaniel et al., 1974), or in 10 mM sodium phosphate/150 mM NaCl, for 2 hours at 0 °C (Kant & Steck, 1973). Either procedure produced qualitatively similar effects on the anisotropy decay. All other procedures were as described according to Matayoshi and Jovin (1991).

Instrumentation and Data Analysis. Samples for which a high-resolution analysis was desirable were measured at multiple times per channel (1024 channels per record). Results such as those in Table I are derived from a global analysis of several such records. The reader is referred to Matayoshi and Jovin (1991) for details. In other measurements such as those reported in Table II and Figures 2 and 3, where only the long-time anisotropy decay behavior was utilized (i.e., millisecond components and r_∞ values), records were obtained at low signal bandwidth and 10 μs per channel. All measurements reported here were taken at 37 °C, in 10 mM sodium phosphate buffer, pH 7.4. As discussed previously, we cannot differentiate between strict rotation versus wobbling motions, nor determine the relative contributions of motions which are global versus local ("segmental" or "breathing") in character. Therefore, in this paper, the term "rotational" mobility, for lack of a more general term, is not meant to convey an assumption of pure uniaxial rotation as the only diffusive mechanism for the observed depolarization of band 3.

Results

Effect of Salt Treatment of Ghosts. When ghosts are prepared by hemolysis in hypotonic phosphate buffers, they retain about two-thirds of the total cellular G3PD (Kant & Steck, 1973). Elution of G3PD from ghosts occurs after incubation in buffers containing saline or higher salt concentrations. Figure 1A demonstrates that salt treatment of ghosts has a significant effect on the $r(t)$ decay of band 3 in ghosts.

In the particular experiment shown, ghosts were stripped of essentially all G3PD by incubation with 0.4 M NaCl (McDaniel et al., 1974). Multiple data records were taken at time resolutions varying from 0.2 to 10 μs per channel, as described in Matayoshi and Jovin (1991). In Figure 1A, the 5 μs per channel record is displayed. Also shown for visual comparison is the $r(t)$ of the control sample. The analyzed results are listed in Table I.

The analysis shows that although the fastest exponential time and amplitude remain constant, the slowest components are altered. As discussed in Matayoshi and Jovin (1991), the control ghosts exhibit a component(s) with apparent correlation time greater than ~5 ms, making quantitation of their amplitude and limiting anisotropy r_∞ imprecise. We find that NaCl treatment of ghosts leads to a reduction of the contribution of these slowest components such that the apparent r_∞ is better defined. The slowest component now has a correlation time of ~3 ms.

The reader will notice that the exact values listed for the control ghosts in Table I differ somewhat from those given in Matayoshi and Jovin (1991). The particular experiment of Figure 1A and Table I utilized ghosts more than 1 day old;

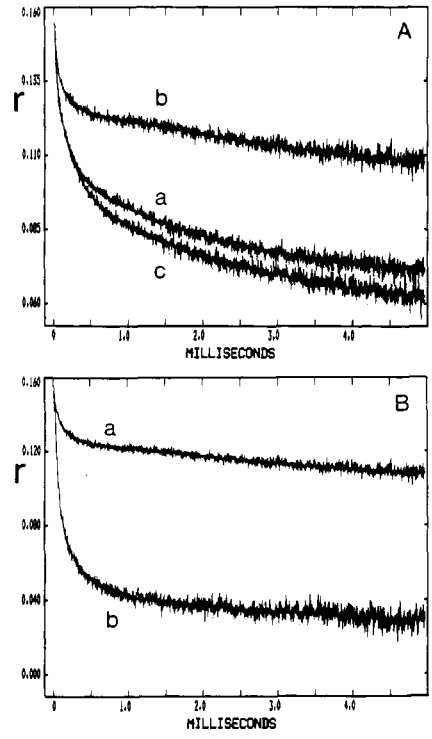

FIGURE 1: Effect of G3PD on the anisotropy decay of eosin-labeled band 3 in ghosts at 37 °C. (A) Curve a, control (untreated); curve b, incubated with 3 μM G3PD; curve c, salt-stripped (G3PD depleted). Results of the anisotropy decay analyses are given in Table I. (B) Curve a, incubated with 3 μM G3PD only; curve b, trypsinized prior to the same G3PD treatment. For the analysis of trypsinized band 3, see Matayoshi and Jovin (1991). In each instance, the data and fit curves are overlaid; all records shown in this figure were taken at 5 μs per channel, high bandwidth, and 1024 channels.

as was mentioned in Matayoshi and Jovin (1991), subtle changes in the $r(t)$ of control ghosts can be detected with age. With aging, the fastest components persist, but there is an increase in r_∞ and an apparent narrowing in the distribution of millisecond anisotropy components (analyzed in fresh ghosts as two apparent discrete components). For these reasons and for simplicity, the comparative analysis in Table I was carried out in terms of three rotational components plus an r_∞ value. Regardless of how the analysis is performed or whether using freshly prepared ghosts or ghosts several days old, the effect of the NaCl treatment on $r(t)$ is qualitatively similar. It is unlikely that this effect is an artifact of the high salt concentration employed, because a similar enhancement of the decay is obtained by simply measuring ghosts in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, and 150 mM NaCl). Furthermore, the effect is reversible in so far as the readdition of G3PD back to NaCl-stripped ghosts affects the $r(t)$ decay in the manner to be described below.

In contrast to the $r(t)$ decay, the NaCl treatment has no effect on $s(t)$. The results and analysis of $s(t)$ data are discussed in Matayoshi and Jovin (1991).

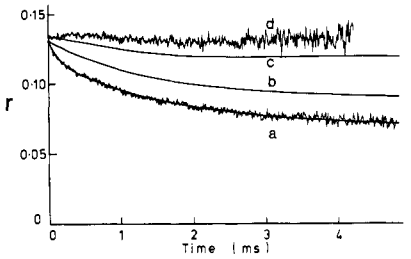

FIGURE 2: Titration of ghosts with G3PD. G3PD was added to ghosts at final concentrations of (a) 0, (b) 1.2, (c) 5.4, and (d) 8.7 μM. Curve a shows both data and fit and curve d the data only. At the two intermediate concentrations, the data are omitted for clarity and only the fitted curves shown. The records were taken at 10 μs per channel, low bandwidth, and 512 channels per record.

Table II: Limiting Anisotropies (r_∞) for Eosin-Labeled Band 3 in the Presence of GPDH and Aldolase[a]

additions	r_∞	additions	r_∞
none (control ghosts)	0.071	8.7 μM G3PD + 0.36 M NaCl	0.081
2.8 μM G3PD	0.097		
5.4 μM G3PD	0.121	2.4 μM aldolase	0.093
8.7 μM G3PD	0.130	5.2 μM aldolase	0.095
8.7 μM G3PD + 1.9 mM NADH, 40 min	0.124	10.2 μM aldolase	0.110
8.7 μM G3PD + 1.9 mM NADH, 80 min	0.099		

[a] Values derived from analysis of records such as those shown in Figures 2 and 3. The ghosts used in this series of measurements were about 48-h old.

Effect of G3PD Binding to Band 3. Previous work (Yu & Steck, 1975; Tsai et al., 1982) has demonstrated that the enzyme G3PD binds specifically to the 41-kDa cytoplasmic domain of band 3. We now show that such binding has a dramatic effect on the anisotropy decay of eosin-labeled band 3 in ghosts.

The addition of micromolar amounts of rabbit muscle G3PD causes a progressive increase in the level of the apparent r_∞ (Figure 2, Table II). At saturating concentrations of G3PD, hardly any decay of $r(t)$ occurs at all. The initial anisotropy $r(0)$ appears to remain constant throughout the titration. The decay measurements in this figure were obtained following addition of G3PD to the cuvette, but the same trend is observed if ghosts are incubated with G3PD and then washed free of excess enzyme, using the low ionic strength buffer (10 mM sodium phosphate, pH 7.4).

The data presented in Figure 2 were taken at low signal bandwidth and are intended to emphasize the longer time behavior. An intermediate situation (i.e., subsaturating concentrations of G3PD) was chosen for more detailed study, and the 5 μs per channel record is shown as curve b in Figure 1A. The analysis indicates (Table I) that while the fastest decay component is left largely unperturbed, the slower components are severely affected. This observation has implications which will be considered under Discussion.

We also find that the $s(t)$ decay is not altered by the binding of G3PD, providing further evidence that the observed effects are due to real changes in the diffusional dynamics [see Discussion in Matayoshi and Jovin (1991)].

At saturating levels of G3PD, the addition of 150 mM NaCl causes an immediate and nearly complete reversal of the G3PD-induced effect (Table II). NADH, a cofactor known to dissociate G3PD from band 3 (Kant & Steck, 1973; Yu & Steck, 1975), is less effective by comparison. When added at a concentration of 2 mM to ghosts saturated with G3PD (37 °C), a slow reversal of $r(t)$ is observed over 30–60 min. However, only a partial reversal to a decay form characteristic of control ghosts is generally obtained. We did not attempt to pursue the nature of the NADH action because the long incubations at 37 °C often proved detrimental to control ghost preparations, presumably as a consequence of the small but significant activity of intrinsic membrane proteases (Tarone et al., 1979; Golovotchenko-Matsumoto et al., 1982). It is possible that the lesser efficacy of NADH, in comparison to NaCl, it related to the formation of ternary complexes of enzyme–nucleotide–band 3 (Kliman & Steck, 1980).

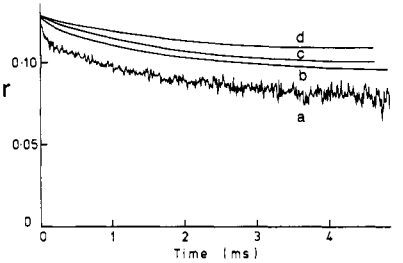

FIGURE 3: Titration of ghosts with aldolase. Aldolase was added to the ghosts at final concentrations of (a) 0, (b) 0.24, (c) 0.52, and (d) 1.02 μM. Curve a shows only the data, whereas for curves b–d only the fits are indicated for clarity. Measurements were taken at 10 μs per channel, low bandwidth, and 512 channels per record.

In addition to the high-affinity sites on band 3, low-affinity membrane sites for G3PD have been identified that may be 20-fold greater in number (McDaniel et al., 1974). We therefore tested the specificity of G3PD action by removing the 41-kDa cytoplasmic fragment of band 3 prior to adding G3PD to ghosts. As shown previously, the cleavage of band 3 by selective trypsinization results in a dramatic enhancement of the decay of $r(t)$ (Nigg & Cherry, 1980; Matayoshi & Jovin, 1991). We find that the addition of G3PD to such trypsinized ghost preparations has no effect on their anisotropy decay (curve b, Figure 1B) and conclude that the influence of G3PD on the $r(t)$ of untreated ghosts is due specifically to binding at sites located on the N-terminal 41-kDa fragment of band 3 molecules.

Effect of Aldolase Binding. To test whether binding-induced "immobilization" of band 3 is possibly a more general phenomenon, some anisotropy decay measurements were also done on ghosts to which exogenous aldolase was added. Aldolase has been shown to bind specifically to the 41-kDa cytoplasmic fragment of band 3 (Strapazon & Steck, 1976, 1977; Murthy et al., 1981).

The anisotropy decay in the millisecond time range of ghosts titrated with aldolase is shown in Figure 3. It is evident that an effect on $r(t)$ is elicited which is qualitatively similar to that obtained after binding by G3PD. At the highest enzyme concentration studied (10 μM), the effect is less complete than was observed for G3PD. The addition of 150 mM NaCl, known to elute aldolase (Strapazon & Steck, 1976), also reversed the effect on $r(t)$.

Discussion

In this study, we have demonstrated that the specific binding of G3PD to the 41-kDa cytoplasmic portion of band 3 dramatically alters the microsecond-to-millisecond anisotropy decay kinetics of eosin-labeled band 3. In the preceding paper (Matayoshi & Jovin, 1991), we discussed evidence which suggests that the widely separated correlation times primarily reflect the rotational mobility of different homo- or hetero-oligomeric complexes of band 3. The present results, therefore, show that the rotational mobility of these complexes can be modulated by the binding of an extrinsic protein.

As discussed in Matayoshi and Jovin (1991), the magnitude of the r_∞ term is determined from the degree of deviation from

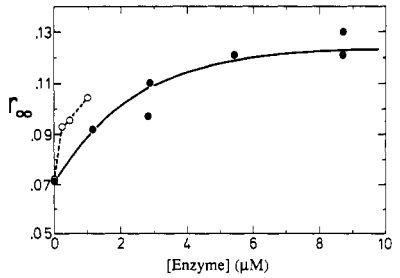

FIGURE 4: Limiting anisotropy (r_∞) of band 3 as a function of G3PD (●) or aldolase (○) concentration. The lines drawn correspond to the simple model discussed in the text, assuming K_a = 1 μM for G3PD.

complete isotropic tumbling of the probe, as well as from the number of species which are immobile on the 10-ms time scale of the experiment. If it is assumed that the binding of each G3PD or aldolase molecule causes the immobilization of one band 3 monomer, then the anisotropy titration data of Figures 2 and 3 can be analyzed as follows: The observed $r(t)$ at any concentration of G3PD would be comprised of two types of contributions, one from immobile band 3–G3PD complexes and the other from band 3 molecules which possess a vacant site for G3PD. The latter group exhibits an $r(t)$ decay presumably identical with that measured for salt-stripped ghosts. Since the total intensity decay $s(t)$ is constant under all treatments, the $r(t)$ from the two groups is simply the mean

$$r(t) = \alpha\{\sum_i r_{0i} \exp(-t/\phi_i) + r_{\infty,f}\} + (1 - \alpha)r_{\infty,b} \quad (1)$$

where α and $1 - \alpha$ are the mole fractions of free and complexed forms of band 3, respectively, and $r_{\infty,f}$ and $r_{\infty,b}$ are the limiting anisotropies of the free and complexed forms of band 3, respectively. At long times, the exponential terms approach zero, and the observed anisotropy ($r_{\infty,obs}$) is given by

$$r_{\infty,obs} = \alpha r_{\infty,f} + (1 - \alpha)r_{\infty,b} \quad (2a)$$

where

$$\alpha = (r_{\infty,b} - r_{\infty,obs})/(r_{\infty,b} - r_{\infty,f}) \quad (2b)$$

Thus, the limiting anisotropy becomes a measure of the binding of G3PD to the membrane. Using values of r_∞ for the free and bound forms (Tables I and II), it is possible to calculate the expected $r_{\infty,obs}$ as a function of G3PD concentration for assumed values of the association constant, K_a. Curves derived from such a computation are shown in Figure 4. Published values of K_a are in the range 10^6–10^8 M^{-1}, depending on conditions of ionic strength and pH (Kant & Steck, 1973; McDaniel et al., 1974). The plots shown in Figure 4 are intended only to show expected trends and not to prove a mechanism for the anisotropy data. It should be noted, for example, that the above calculation does not consider the existence of any low-affinity G3PD binding sites on the membrane, which McDaniel et al. (1974) estimate as outnumbering the high-affinity sites by 20-fold or more. Also not included in the calculation is an experimental factor accounting for the possibility that the true concentration of free tetrameric G3PD is lower than assumed, because of its tendency to form aggregates at low ionic strength and elevated temperature.

The binding sites for G3PD and aldolase lie within the first 23 N-terminal residues of band 3 (Murthy et al., 1981; Tsai et al., 1982). Whether these sites are identical, overlapping, or distinct has not yet been fully resolved. The binding in both cases seems to be mainly electrostatic in nature, as judged by its strong dependence on ionic strength and pH. Furthermore, Murthy et al. (1981) have shown that the amino acid composition at the amino-terminal end of band 3 is highly acidic, the first 31 residues containing 16 negatively charged residues (Asp or Glu), no basic residues, and a blocked α-amino group. These findings make it unlikely that the polypeptide chain is folded up, and indeed Appell and Low (1981) have demonstrated that the isolated 41-kDa fragment of band 3 behaves hydrodynamically as an elongated dimeric particle of frictional ratio 1.6, implying an axial ratio perhaps as large as 10.

The mechanism by which band 3 is rendered rotationally immobile following G3PD or aldolase binding is unclear. The ill-defined model of one G3PD/one immobilized band 3, chosen above as a starting point for analysis, would appear to require the triggering in some manner of a large conformational change in band 3 by which band 3–protein and band 3–lipid interactions are greatly perturbed. However, as discussed in the preceding paper for the trypsinization of the 41-kDa cytoplasmic fragment (Matayoshi & Jovin, 1991), the constancy of $s(t)$ and $r(0)$ in the presence vs absence of G3PD argues against a large conformational change. Moreover, this type of model does not explain the greater persistence of the component with the shortest correlation time [which in Matayoshi and Jovin (1991) we attribute to band 3 dimers] even at concentrations of G3PD where substantial immobilization of band 3 has been induced, i.e., where r_∞ has become large (Table I). Although this result could be rationalized if G3PD were bound with higher affinity to the larger aggregates of band 3, this seems unlikely because independent biochemical evidence indicates the presence of just one set of high-affinity G3PD sites (Kant & Steck, 1973; McDaniel et al., 1974). An alternative type of model which better accounts for our results is one in which the immobilization is brought about only following the cross-linking of band 3 aggregates by G3PD, and not merely by its binding to a single band 3 molecule. In other words, the immobilization (as monitored by the magnitude of r_∞) would be more efficient for preexisting small clusters of band 3 because a single or a few cross-links between them, creating a much larger aggregate, could result in all members now being detected as "immobile". By comparison, it would require many cross-links of isolated dimers to reach the size of an apparently immobile aggregate (10-ms time scale). According to this model, bound G3PD would be equally distributed among species of all sizes, and an approximate linear dependence of the r_∞ value upon total bound G3PD (as was analyzed above) might still be observed. Of considerable interest is the implication that the binding of G3PD to isolated dimers has little effect on their rotational correlation time.

The stoichiometry of one enzyme tetramer per band 3 monomer at saturation, for either G3PD or aldolase (Yu & Steck, 1975; Strapazon & Steck, 1976), might appear to argue against a cross-linking-type mechanism. However, binding experiments are not precise enough to rule out cross-linking, since it would require little deviation from an exact 1:1 stoichiometry in order to cross-link preexisting aggregates with one another. Furthermore, if the 41-kDa segment protrudes as a dimer 250 Å in length away from the membrane as proposed by Low (1986), since the binding sites for the glycolytic enzymes are located on the extreme N-terminal end, the cross-linking of neighboring band 3 oligomers would be possible even when their hydrophobic domains are at some distance apart (e.g., as when restricted lateral diffusion does not permit collisional contact). Beth et al. (1981) reported that spin-labeled G3PD bound to band 3 in ghosts exhibits an effective isotropic correlation time of 20 μs. This result appears at first glance to be at variance with our findings. However, their experiments were conducted at G3PD/band 3 stoichiometries no greater than those normally present in ghosts

prepared in hypotonic phosphate buffers. (Hence, their result must be considered with respect to the anisotropy decay of band 3 in "control" ghosts.) Furthermore, the ST-EPR technique, in its present state of the art, does not permit easy assessment of the degree of motional heterogeneity or anisotropy. Their 20-μs correlation time might derive from the restricted diffusion of a tethered 82-kDa dimer of the band 3 cytoplasmic segment, complexed with G3PD.

The immobilization of band 3 caused by G3PD or aldolase is of further relevance to the question of hemoglobin binding to band 3 in the intact cell. Hemoglobin competes with G3PD for binding sites on band 3 in ghosts and has been shown to interact with the same N-terminal 23 residues (Shaklai et al., 1977; Salhany & Shaklai, 1979; Murthy et al., 1984). It also undergoes oxidative disulfide cross-linking to band 3 in ghosts with 1:1 stoichiometry (Sayare & Fikiet, 1981). Measurements of fluorescence energy transfer in intact cells between DIDS-labeled band 3 and cytoplasmic hemoglobin are consistent with hemoglobin being located on or in the immediate vicinity of band 3 (Eisinger et al., 1982). Studies by various investigators indicate that hemoglobin binds either to ghosts or to the cytoplasmic peptide fragment of band 3 with a K_a of only $\sim 10^3$–10^4 M^{-1} at physiological pH (Fung, 1981; Cassoly, 1983; Walder et al., 1984; Chetrite & Cassoly, 1985). Nonetheless, considering the extremely high concentration of hemoglobin within the erythrocyte (5 mM tetramer) and the normal concentrations of O_2 and 2,3-diphosphoglycerate, Chetrite and Cassoly (1985) estimated that about half of the band 3 per cell interacts with hemoglobin. If band 3–hemoglobin complexes are, in fact, present to this extent, the results of Matayoshi and Jovin (1991) on intact cells and hemoglobin-free ghosts imply that the rotational mobility of band 3 is not significantly affected by the presence of hemoglobin at its cytoplasmic pole. The absence of an effect is not difficult to reconcile with the G3PD- and aldolase-induced immobilization if the cross-linking mechanism proposed above is correct. In other words, the subunits of hemoglobin tetramers, in addition to their much lower affinity for band 3, might not be juxtaposed favorably to permit cross-linking of band 3 oligomers. The lower efficacy of band 3 immobilization by aldolase in comparison to G3PD might also be the result of a relative subunit orientation that is not optimal for cross-linking.

Whether the rotational diffusion of band 3 per se has any physiological significance remains an open issue. In the polarized phosphorescence experiments, we are, a priori, studying band 3 in an anion transport-inhibited state. However, it has become clear that the rapid exchange of anions may be only one of several roles that band 3 serves in the erythrocyte. The notion of multiple roles for band 3 may be inferred from the number of copies of band 3 per cell, which far exceeds that of any individual glycolytic or cytoskeletal membrane protein for which a 1:1 binding stoichiometry has been shown. As a specific interaction site for several glycolytic enzymes whose activity is altered upon binding (Tsai et al., 1982; Strapazon & Steck, 1977; Higashi et al., 1979; Jenkins et al., 1985), band 3 may be involved in the regulation of cellular metabolism (Gillies, 1982). Furthermore, band 3 may constitute a common locus for the binding of cytoskeletal and cytoplasmic proteins, and thus play a central role in coordinating cellular flexibility and motility with other functions (Salhany & Gaines, 1981). In this and the preceding paper, we have demonstrated that the rotational diffusion of band 3 is sensitive to interactions with extrinsic proteins, thereby providing another example for the utility of the underlying biophysical technique in the elucidation of cellular surface events at the molecular level.

Registry No. G3PD, 9001-50-7; aldolase, 9024-52-6.

References

Appell, K. C., & Low, P. S. (1981) *J. Biol. Chem. 256*, 11104–11111.

Beth, A. H., Balasubramanian, K., Wilder, R. T., Venkataramu, S. D., Robinson, B. H., Dalton, L. R., Pearson, D. E., & Park, J. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4955–4959.

Brindle, K. M., Campbell, I. D., & Simpson, R. J. (1982) *Biochem. J. 208*, 583–592.

Cassoly, R. (1983) *J. Biol. Chem. 258*, 3859–3864.

Chetrite, G., & Cassoly, R. (1985) *J. Mol. Biol. 185*, 639–644.

Eisinger, J., Flores, J., & Salhany, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 408–412.

Fossel, E. T., & Solomon, A. K. (1981) *Biochim. Biophys. Acta 649*, 557–571.

Fung, L. W. M. (1981) *Biochemistry 20*, 7162–7166.

Gillies, R. J. (1982) *Trends Biochem. Sci. 7*, 41–42.

Golovtchenko-Matsumoto, A. M., Matsumoto, I., & Osawa, T. (1982) *Eur. J. Biochem. 121*, 463–467.

Higashi, T., Richards, C. S., & Uyeda, K. (1979) *J. Biol. Chem. 254*, 9542–9550.

Jenkins, J. D., Madden, D. P., & Steck, T. L. (1984) *J. Biol. Chem. 259*, 9374–9378.

Jenkins, J. D., Kezdy, F. J., & Steck, T. L. (1985) *J. Biol. Chem. 260*, 10426–10433.

Kant, J. A., & Steck, T. L. (1973) *J. Biol. Chem. 248*, 8457–8464.

Kliman, H. J., & Steck, T. L. (1980) *J. Biol. Chem. 255*, 6314–6321.

Low, P. S. (1986) *Biochim. Biophys. Acta 864*, 145–167.

Maretzki, D., Reimann, B., & Rapoport, S. M. (1989) *Trends Biochem. Sci. 14*, 93–96.

Masters, C. (1989) *Trends Biochem. Sci. 14*, 361.

Matayoshi, E. D., Corin, A. F., Zidovetzki, R., Sawyer, W. H., & Jovin, T. M. (1983) in *Proceedings of the FEBS Symposium Konstanz, 1982: Mobility and Recognition in Cell Biology* (Sund, H., & Veeger, C., Eds.) pp 119–134, Walter de Gruyter, Berlin.

Matayoshi, E. D., & Jovin, T. M. (1991) *Biochemistry* (preceding paper in this issue).

McDaniel, G. F., Kirtely, M. E., & Tanner, M. J. A. (1974) *J. Biol. Chem. 249*, 6478–6485.

Murthy, S. N. P., Liu, T., Kaul, R. K., Kohler, H., & Steck, T. L. (1981) *J. Biol. Chem. 256*, 11203–11208.

Murthy, S. N. P., Kaul, R. K., & Kohler, H. (1984) *Hoppe-Seyler's Z. Physiol. Chem. 365*, 9–17.

Nigg, E. A., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702–4706.

Premachandra, B. R. (1986) *Biochemistry 25*, 3455–3462.

Rich, G. T., Pryor, J. S., & Dawson, A. P. (1985) *Biochim. Biophys. Acta 817*, 61–66.

Salhany, J. M., & Shaklai, N. (1979) *Biochemistry 18*, 893–899.

Salhany, J. M., & Gaines, K. C. (1981) *Trends Biochem. Sci. 6*, 13–15.

Salhany, J. M., Cordes, K. A., & Gaines, K. C. (1980) *Biochemistry 19*, 1447–1454.

Sayare, M., & Fikiet, M. (1981) *J. Biol. Chem. 256*, 13152–13158.

Shaklai, N., Yguerabide, J., & Ranney, H. M. (1977) *Biochemistry 16*, 5593–5597.

Solti, M., Bartha, F., Halasz, N., Toth, G., Sirokman, F., &

Friedrich, P. (1981) J. Biol. Chem. 256, 9260-9265.
Steck, T. L. (1978) J. Supramol. Struct. 8, 311-324.
Strapazon, E., & Steck, T. L. (1976) Biochemistry 15, 1421-1424.
Strapazon, E., & Steck, T. L. (1977) Biochemistry 16, 2966-2971.
Tarone, G., Hamasaki, N., Fukuda, M., & Marchesi, V. T. (1979) J. Membr. Biol. 48, 1-12.
Tsai, I., Murthy, S. N. P., & Steck, T. L. (1982) J. Biol. Chem. 257, 1438-1442.
Walder, J. A., Chatterjee, R., Steck, T. L., Low, P. S., Musso, G. F., Kaiser, E. T., Rogers, P. H., & Arnone, A. (1984) J. Biol. Chem. 259, 10238-10246.
Yu, J., & Steck, T. L. (1975) J. Biol. Chem. 250, 9176-9184.

Biochemical Analysis of the Ligand for the *neu* Oncogenic Receptor†

Yosef Yarden* and Elior Peles
Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel
Received June 21, 1990; Revised Manuscript Received August 15, 1990

ABSTRACT: The *neu* protooncogene (also called HER2 and c-erbB2) encodes a cell-surface tyrosine kinase structurally related to the receptor for the epidermal growth factor (EGF). We have previously reported that a candidate ligand for the *neu* receptor is secreted by *ras*-transformed fibroblasts. Biochemical analyses of the *neu* stimulatory activity indicate that the ligand is a 35-kDa glycoprotein that is heat stable but sensitive to reduction. The factor is precipitable by either high salt concentrations or acidic alcohol. Partial purification of the molecule by selective precipitation, heparin–agarose chromatography, and gel filtration in dilute acid resulted in an active ligand, which is capable of stimulating the protooncogenic receptor but is ineffective on the oncogenic *neu* protein, which is constitutively active. The purified fraction, however, retained the ability to stimulate also the related receptor for EGF, suggesting that these two receptors are functionally coupled through a bidirectional mechanism. Alternatively, the presumed ligand interacts simultaneously with both receptors. The presented biochemical characteristics of the factor are expected to enable a completely purified factor with which to explore these possibilities.

The enzymatic catalysis of the transfer of a phosphate group to tyrosyl residues of polypeptides appears to be uniquely associated with the control of cell growth (Hunter, 1990). Most of the known mammalian tyrosine kinases are encoded by genes that are potoentially oncogenic. Release of the transforming potential is usually achieved through structural alterations that result in deregulated catalysis of tyrosine phosphorylation. Unlike that of the soluble tyrosine kinases, the mechanism of regulation of transmembrane tyrosine kinases is relatively well understood. Binding of peptide ligands to the extracellular domain of the corresponding receptor allosterically elevates the intrinsic enzymatic activity of the kinase [reviewed by Yarden and Ullrich (1988)]. It is therefore important that a growing list of membrane tyrosine kinases with landmarks of receptor molecules have no identified ligands. This list currently includes the genes *neu* (Coussens et al., 1985; Bargmann et al., 1986a; Yamamoto et al., 1986), *kit* (Yarden et al., 1987), *ros* (Neckameyer et al., 1988), *met* (Park et al., 1987), *trk* (Martin-Zanca et al., 1986), *trkB* (Klein et al., 1989), *ret* (Takashi & Cooper, 1987), and *eph* (Hirai et al., 1987). Potentially, each putative receptor has a cognate ligand that is involved in cell regulation. The identification of these hypothetical molecules through "reverse" biochemistry of the cognate receptor molecules is therefore a promising challenge.

The *neu* protooncogene (also called HER2 and c-erbB2) is a prototypic putative receptor for a still unknown ligand. The encoded protein is a 185-kDa transmembrane glycoprotein whose extracellular domain is highly homologous to the ligand-binding portion of the epidermal growth factor receptor, whereas the cytoplasmic part carries mostly tyrosine kinases sequences. A carcinogen-induced point mutation within the transmembrane stretch of amino acids (Bargmann et al., 1986b) releases the oncogenic potential of the presumed receptor through a mechanism that involves elevated kinase activity (Bargmann & Weinsberg, 1988; Stern et al., 1988; Yarden, 1990). Alternatively, overexpression of the protooncogenic human receptor leads to phenotypic transformation of cultured cells (DiFiore et al., 1987; Hudziak et al., 1987). This observation may be relevant to some human adenocarcinomas that display amplification or overexpression of the *neu* gene (Slamon et al., 1987; Varley et al., 1987; Venter et al., 1987; Zhou et al., 1987; Tal et al., 1988).

Despite the structural resemblance, p185^{neu} does not function as a receptor for the epidermal growth factor (EGF)[1] or the related transforming growth factor α (TGFα; Stern et al., 1986). Yet, the homologous epidermal growth factor receptor, upon ligand binding, interacts with p185^{neu} to increase tyrosine phosphorylation of the latter protein (Stern & Kamps, 1988; King et al., 1988; Kokai et al., 1988). In addition, the catalytic activity of the *neu* receptor can be allosterically modulated by the binding of monoclonal antibodies to its extracellular part (Yarden, 1990). On the basis of the structural and functional similarities between p185^{neu} and other receptors for growth factors, we assumed that a still unknown natural ligand

† This work was supported by grants from The Fund for Basic Research, administered by the Israeli Academy of Sciences and Humanities, and the Israel Cancer Research Fund and by Grant 1R01 CAS1712 from the National Institutes of Health.

[1] CNBr, cyanogen bromide; EGF, epidermal growth factor; EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; TGFα, transforming growth factor α; SDS, sodium dodecyl sulfate.

0006-2960/91/0430-3543$02.50/0