

Self-association of Glucagon As Measured by the Optical Properties of Rhodamine 6G*

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The fluorescence of rhodamine 6G is completely quenched in glucagon solutions in 0.6 M K_2HPO_4 at pH 10.6. The absorption of rhodamine 6G is red-shifted by the same reaction. A single rhodamine 6G molecule appears to be bound to a hydrophobic patch in the center of the trimer of glucagon. Since the glucagon monomer has almost no organized structure this site exists only in the associated trimer form of glucagon. The self-association of glucagon to the trimer has been determined from the variation in rhodamine 6G fluorescence and absorption measured over a 60-fold range of dye concentration. The self-association constant agrees with values determined by other methods in the absence of dye. The binding isotherms of rhodamine 6G to glucagon shift with glucagon concentration and exhibit negative cooperativity.

are largely between hydrophobic residues in both trimer configurations. One important difference between the two configurations is that the residues neighboring the region around the intersection of the 3-fold axes are completely different, arginines and aspartic acids in one configuration and leucine, valine, and phenylalanine in the other. Since the central region of the latter configuration is comprised of 9 nonpolar residues we tested several fluorescent probes for binding to glucagon. Rhodamine 6G was bound much stronger than other probes and we have calculated the self-association constant of glucagon from the binding data of this dye. The experimental conditions were kept similar to those used to measure this constant by circular dichroism (11) and sedimentation equilibrium in order to compare the various methods.²

MATERIALS AND METHODS

Crystalline glucagon was obtained from Elanco Products Co. (Division of Eli Lilly and Co.) by Dr. M. Rodbell (National Institutes of Health) and from Sigma. Glucagon was dissolved in the K_2HPO_4 solutions at room temperature. The pH of the solutions was adjusted with KOH and solutions were centrifuged to clarify completely. The concentration of glucagon was determined using the specific absorbance, at 278 nm, $\epsilon_{1\%}^{1\text{cm}} = 23.7$ (12). The isosbestic point for tyrosine ionization in glucagon is at 278 nm. ANS and acridine orange were obtained from Eastman Kodak, Rhodamine 6G from National Aniline, and Dns glycine from Sigma. Glass-distilled water was used throughout and all other chemicals were reagent grade.

A Perkin-Elmer MPF-3 fluorescence spectrophotometer was used for all fluorescence and polarization measurements. This instrument has a temperature-controlled cell holder and all measurements were made at 25°. Rhodamine 6G was excited at 480 nm and its emission was monitored at 548 nm. ANS was excited at 360 nm and monitored at 480 nm. The optical density at the excitation wavelength of all solutions used for fluorescence was less than 0.08 in order to minimize inner filter effects.

Difference absorbance changes of rhodamine 6G were measured at 542 nm using a Gilford modified Beckman DU spectrophotometer. This instrument has a temperature-controlled cell holder and all measurements were made at 25°.

The data have been analyzed according to a monomer n -mer self-association model where the n -mer has m identical independent dye binding sites and the monomer does not bind dye. For this model the total macromolecular concentration can be expressed as

$$[C_t] = [C_m] + n K_a (1 + K_b [L])^m [C_m]^n \quad (1)$$

where $[C_m]$ is the monomer concentration, n is the degree of polymerization, K_a is the self-association constant in the absence of ligand, K_b is the dye-binding constant, $[L]$ is the free ligand, and

Since Monod *et al.* (1) introduced the concept of allostery to explain cooperativity in enzyme kinetics many studies have confirmed that small molecules (effectors) which bind to the enzyme can regulate enzyme activity. In those allosteric systems in which only the conformation of the enzyme is modified the effector binding or enzyme activity is independent of the concentration of the enzyme. With other allosteric enzymes, however, the binding or activity varies with the concentration of the enzyme. In these cases the binding of the small molecule is coupled to the subunit association equilibria of the enzyme and will influence the relative amounts of the various protein species co-existing in solution (2-5). Either the effect of protein concentration on the ligand-binding isotherms or the effect of ligand concentration on subunit association can be used to study systems of this type (4).

In addition to physiological effectors which bind to enzymes or proteins and shift equilibria between molecular species, fluorescent probes have been shown to produce similar effects (6, 7). An example is the negative and positive cooperativity observed in the binding of ANS,¹ respectively, to luteinizing hormone (8) and chorionic gonadotropin (9).

Glucagon crystallizes as an oligomer of trimers with two 3-fold axes (10). The interactions between the monomer chains

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¹ The abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; Dns, 5-dimethylaminonaphthalene-1-sulfonyl.

² S. Formisano, M. L. Johnson, and H. Edelhoach, unpublished data.

$[C_t]$ is the total concentration of macromolecule expressed in monomer units.

The concentration of bound ligand $[L_b]$ can be expressed in terms of the total ligand, $[L_t]$, as

$$[L_b] = [L_t] - [L] = m K_a K_b [L](1 + K_b[L])^{m-1} [C_m]^n \quad (2)$$

This model has been used previously to evaluate the binding of ANS to luteotropic hormone (8) and chorionic gonadotropin (9). These two homologous hormones have the same function but bind different amounts of ANS.

The fluorescence of the ANS glucagon mixtures was taken to be proportional to the amount of ANS bound since free ANS has a very low quantum yield, *i.e.*

$$\text{Fluorescence}_{\text{ANS}} = Z [L_b] \quad (3)$$

where Z is a constant which includes both the quantum yield of the bound ANS and the gain of the fluorometer.

The difference change in optical density, ΔA , of the rhodamine 6G glucagon mixtures was also taken to be proportional to the amount of bound dye,

$$\Delta A = \Delta \epsilon [L_b] \quad (4)$$

where $\Delta \epsilon$ is the difference in molar extinction coefficient between the bound and free dye.

The fluorescence of rhodamine 6G in glucagon solutions relative to that in the absence of glucagon was taken as

$$\text{Relative fluorescence} = 1 - Q L_b/L_t \quad (5)$$

where Q is the fractional amount of quenching, *i.e.* $1 - \text{ratio of fluorescence intensities of bound to free dye}$.

The bound ligand concentration $[L_b]$, the free ligand $[L]$, and monomer concentration $[C_m]$ can be evaluated from the total concentrations, $[C_t]$ and $[L_t]$ by finding a simultaneous root of Equations 1 and 2.

Recently it was found by cross-linking experiments with dimethyl suberimidate that the associated species of glucagon is a trimer; no dimers or hexamers were found (13). It is also known that glucagon is a trimer in its crystalline state (10). Consequently, the degree of polymerization, n , was taken to be three.

Values of the equilibrium constants, Z , $\Delta \epsilon$, and Q were determined by least squares fits of the experimental data to the functional forms described above. These "best fits" were accomplished with an on-line modeling program, MLAB, developed at National Institutes of Health (14). This program utilizes the Marquardt-Levenberg algorithm to do a least squares fit of data to an arbitrary equation (15). Reported standard errors of fitted parameters correspond to approximately 1 S.D.

RESULTS

We have recently reported the self-association constants of glucagon at pH 10.6 in 0.2 M and in 0.76 M K_2HPO_4 (11).² We

have been able to determine this equilibrium constant, at 0.6 M K_2HPO_4 , by using a fluorescent dye, rhodamine 6G, which binds to the associated form of glucagon. In order to extend the range of dye concentrations used, and consequently improve the precision of the determination of the self-association constant, both fluorescence and absorption measurements have been made with this dye. The two methods are complementary since fluorescence measurements are possible at much lower dye concentrations than absorption.

The fluorescence of ANS (4.7 μM) was measured with increasing concentration of glucagon at pH 10.6 in 0.60 M phosphate. The extent of ANS binding was too small to analyze for the self-association constant. The pH was therefore raised to 11.2 in order to increase the solubility of glucagon in 0.60 M phosphate. Glucagon is soluble at least to 1500 μM at these conditions. The increase in fluorescence of ANS, as a function of glucagon concentration, is shown in Fig. 1 at two concentrations of ANS, *i.e.* 4.7 and 14.0 μM .

The polarization of ANS fluorescence was measured with increasing glucagon concentration at both levels of ANS. The polarization was independent of ANS binding from about 25 to 75% saturation and equal to 0.160 ± 0.005 . If ANS were bound to monomeric glucagon there would be a substantial change in polarization with ANS binding since the relaxation time of the monomer should be significantly smaller than that of the trimer. It may be concluded therefore that ANS does not bind to monomeric glucagon. The model of ANS binding to glucagon has therefore not included binding to monomer.

Analysis of both ANS fluorescence curves gives a self-association constant of $6.8 \times 10^6 \pm 2.1 \times 10^6 \text{ M}^{-2}$ independent of the number of ANS sites. The binding constant for ANS was $5.3 \times 10^3 \pm 0.7 \times 10^3 \text{ M}^{-1}$ assuming one site per trimer. The number of ANS sites could not be determined since only a very small fraction of glucagon trimers bind ANS in our experiments. The self-association obtained at 11.2 in 0.6 M phosphate is almost 1 order of magnitude smaller than at pH 10.6 in the same buffer.

When glucagon was added to a solution of rhodamine 6G, at pH 10.6 in 0.60 M phosphate, the fluorescence was strongly quenched. The quenching of rhodamine 6G fluorescence between 1.08 and 6.51 μM dye concentration is shown in Fig. 2. An analysis for the binding of 1 dye molecule to the glucagon

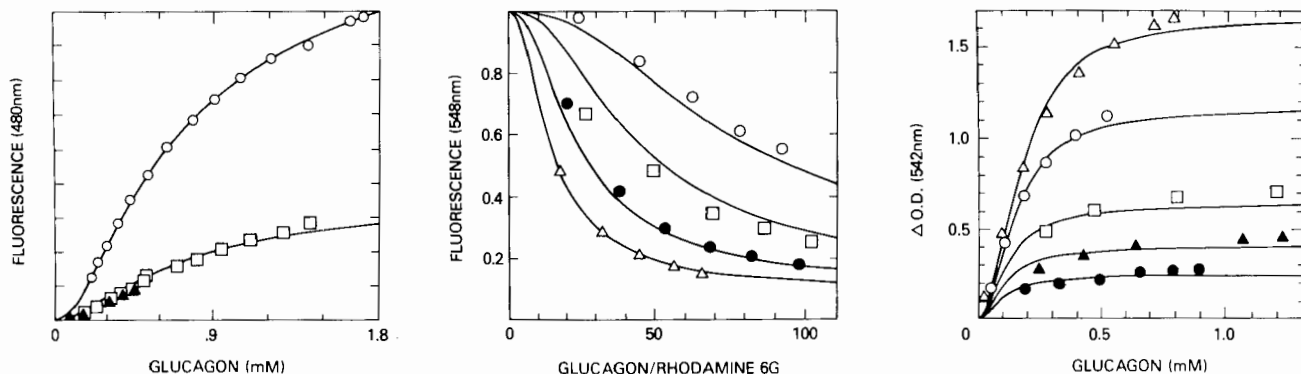


FIG. 1 (left). The effect of glucagon on the fluorescence intensity of ANS at 480 nm at pH 11.2, 0.60 M K_2HPO_4 ; \blacktriangle , \square = 4.7 μM ; \circ = 14.0 μM ANS. Fluorescence is expressed in arbitrary units.

FIG. 2 (center). Relative fluorescence of rhodamine 6G as a function of mole ratio of glucagon and rhodamine 6G. The solid lines are the "best fit," as described in the text, assuming that one rhodamine 6G is bound to the trimeric species and using Equations 1, 2, and 5. Rhodamine 6G concentrations were 1.08 μM (\circ), 1.94 μM (\square), 3.80

μM (\bullet), and 6.51 μM (\triangle).

FIG. 3 (right). Difference absorbance as a function of the concentration glucagon and rhodamine 6G. The solid lines are the "best fit" to this data, as described in text, using Equations 1, 2, and 4, and assuming that one rhodamine 6G is bound to the trimeric species. The rhodamine 6G concentrations were 9.1 μM (\bullet), 14.8 μM (\blacktriangle), 23.0 μM (\square), 41.7 μM (\circ), and 59.5 μM (\triangle).

trimer gives a self-association constant, K_a , of $3.9 \times 10^7 \pm 3.4 \times 10^7 \text{ M}^{-2}$, a dye binding constant, K_b , of $103 \times 10^3 \pm 45 \times 10^3 \text{ M}^{-1}$ and a fluorescence quenching, Q , of $93\% \pm 5\%$. It appears that rhodamine 6G fluorescence is totally quenched by binding to glucagon trimers.

In order to reduce the error in the constants the range of rhodamine 6G concentration was increased by exploiting the change in its absorption on binding to glucagon. The peak of the absorption spectrum of rhodamine 6G is red-shifted from 527 to 546 nm when bound to glucagon. The change in optical density of rhodamine 6G between 9.1 and 59.5 μM dye and 0.0 to 1.3 mM glucagon is shown in Fig. 3. Analysis of these data by the same model ($m = 1$) as used for the fluorescence data gave $6.4 \times 10^7 \pm 3.3 \times 10^7 \text{ M}^{-2}$ for K_a and $88 \times 10^3 \pm 13 \times 10^3 \text{ M}^{-1}$ for K_b . The molar difference extinction was $28 \times 10^3 \pm 2 \times 10^3 \text{ M}^{-1}$ at 542 nm. Consequently dye-binding data measured over a 60-fold range of dye concentration by two independent methods give similar constants for self-association and dye binding. In order to determine the best value of these two constants the fluorescence and absorption data were fit simultaneously giving the two types of data equal weight. This simultaneous analysis gave a self-association constant of $4.8 \times 10^7 \pm 1.5 \times 10^7 \text{ M}^{-2}$ and a dye binding constant of $94 \times 10^3 \pm 10 \times 10^3 \text{ M}^{-1}$. The marked improvement in the standard

errors of the computed constants is a reflection of the wider range of dye concentration used in the simultaneous analysis. From an error analysis of the data it is evident that only one rhodamine 6G is bound. The assumption that 2 dye molecules are bound to the trimer caused the variance of the "best fit" to increase by a factor of 2.5 as compared to that for one dye bound. The variance ratio (F - statistic) for a 1% probability is approximately 2.0; consequently, a value of 2.5 corresponds to a very low probability. The self-association constant determined by the fluorescence and absorption changes in rhodamine 6G agrees with values obtained by circular dichroism and sedimentation equilibrium (11).²

The binding of rhodamine 6G to glucagon can also be analyzed as a cooperative phenomenon. We have calculated the dye-binding isotherms over a 10^4 range of glucagon concentration from the equilibrium constants given in the preceding paragraph: $K_a = 4.8 \times 10^7$, $K_b = 9.4 \times 10^4$. The binding curve and Hill constant (Figs. 4 and 5) for 10^{-2} M glucagon are very close to being noncooperative since glucagon is mostly trimer at this concentration. At 10^{-3} M glucagon the binding curve is shifted slightly to higher dye concentration and the slope decreases slightly, indicating minor negative cooperativity. With decreasing glucagon concentration the binding curves are increasingly shifted to higher dye concentration and the slopes (at $\bar{Y} = 0.5$) decrease further. The degree of negative cooperativity of this system is seen to depend strongly on the glucagon concentration. Moreover the "Hill constant" for this system becomes meaningless since it decreases with either increasing binding or with decreasing protein concentration.

DISCUSSION

The modification of enzyme activity by allosteric effectors has been extensively documented since Monod *et al.* proposed their model to explain sigmoidal activity curves, *i.e.* subunit cooperativity (1). The models of Monod *et al.* (1) and Koshland *et al.* (16) treat only protein isomerization processes due to ligand binding. It was demonstrated by Nichol *et al.* (17) that the binding of small molecules could also shift the equilibrium between associated states of proteins. In the latter case binding curves reveal cooperativity and depend on protein concentration. Frieden was able to show these effects by measuring both the binding curve of GTP to glutamate dehydrogenase and the change in the weight average molecular weight of glutamine dehydrogenase with binding (2). When he used an activator of the enzyme, *i.e.* ADP, he observed that it bound more strongly to the higher polymeric species, in contrast to the inhibitor GTP which shows the opposite effect. In a subsequent review of this subject Frieden indicated that the non-Michaelis-Menten behavior of numerous other enzymes toward their substrates, etc. could be explained by differences in the affinity of the small molecules to the different species of the enzyme in equilibrium with each other (18). Recent studies have afforded data supporting such mechanisms.

Perhaps the most definitive demonstration of the effects of ligand binding on protein subunit association is the recent work of Ip and Ackers (19). They have observed very large differences in all the thermodynamic parameters (ΔG° , ΔH° , and ΔS°) between oxygenated and unliganded hemoglobin A for the dimer to tetramer association.

Fluorescent molecules have also been used to measure association reactions. The fluorescence of the coenzyme, NADH, was used to measure the equilibrium constant between the monomer and dimer form as well as the binding

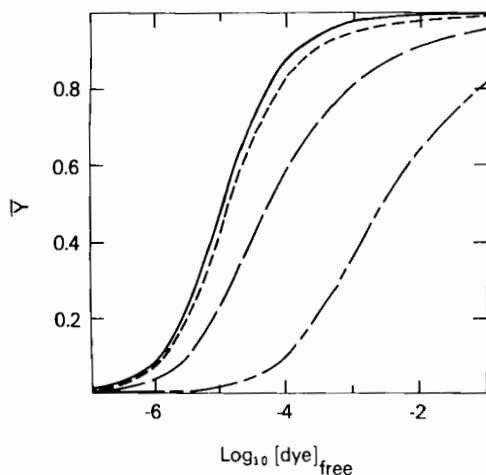


FIG. 4. The effect of glucagon concentration on the binding isotherm of rhodamine 6G. Curves were calculated using $K_a = 4.8 \times 10^7$, $K_b = 9.4 \times 10^4$, and glucagon concentrations of 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M from left to right. \bar{Y} is the fractional saturation of sites.

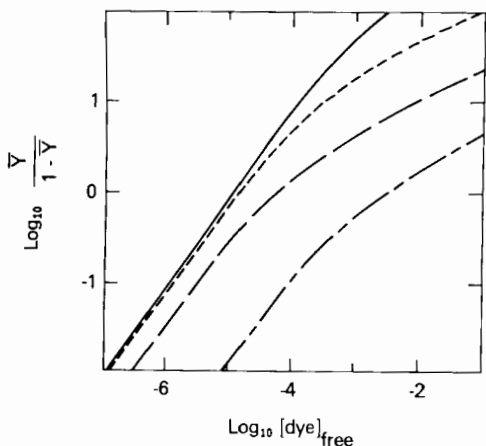


FIG. 5. Hill plots corresponding to Fig. 4. Glucagon concentrations are 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} M (left to right).

constants to the monomer and dimer of malic dehydrogenase (3). The effect of AMP and $MnCl_2$ on the rates of association of phosphorylase *b* dimer to tetramer was measured by the enhancement in fluorescence of 2-methylanilino-naphthalene-6-sulfonate (6). The dimer form of D-amino acid oxidase was increased by ANS but decreased by 4-monobenzoylamido-4'-aminostilbene-2,2'-disulfonate (7). Both compounds inhibit the activity of the enzyme by competing for the substrate or FAD. However, equilibrium constants for the monomer-dimer equilibrium or dye bonding were not obtained.

There have been relatively few studies where protein self-association constants have been independently evaluated in the presence and absence of ligands. The self-association of glucagon has been reported by us recently and by others earlier. It is now well established by chemical modification using cross-linking reagents and by x-ray diffraction that the trimer is the only associated species. Consequently the model used to evaluate the rhodamine 6G (and ANS) binding curves is known except for the number of dye molecules bound. It is evident from the ANS polarization data that ANS is not bound to the monomer. In this case one has only to consider dye binding to the trimer. The number of ANS molecules bound to the trimer could not be evaluated since the binding is too weak. Binding of rhodamine 6G was much stronger and a much better fit was obtained with one dye as compared with 2 dye molecules bound per trimer. It has been found by fluorescence and polarization measurements that Dns glycine and acridine orange are also bound to glucagon at pH 10.6, 0.60 M phosphate but more weakly than rhodamine 6G; consequently the binding of these two dyes was not studied in detail.

It is not known which of the two trimer species reported from x-ray studies of glucagon crystals by Sasaki *et al.* is present in solution (10). It seems likely that hydrophobic dyes would react preferentially, if not exclusively, with the trimer form having its center occupied by nonpolar residues rather than by the highly charged ones. Since the self-association constant measured by rhodamine 6G agrees with that measured by non-probe methods, it seems likely that the form with the hydrophobic core is the species present in solution. Moreover hydrophobic interactions among the nonpolar residues would contribute to the stability of this conformation. In the other conformation, it is not clear whether attractive or repulsive electrostatic interactions would dominate between

the 2 arginine and 1 aspartate residue (per monomer) situated in the center of the trimer.

Once a model is established it is possible to use the properties of the probe to measure the thermodynamics or kinetics of self-association systems. This can be an important technique to study some of the more complex self-associating systems, *i.e.* microtubules, microfilaments, etc., particularly at very low concentrations where other techniques are not applicable. It may also be possible because of the sensitivity of fluorescence measurements to work at sufficiently low concentrations of protein so that lower molecular weight aggregates may be observed. The latter equilibria should be more amenable to thermodynamic analysis than that of the end product of the reaction, *i.e.* the fibril or filament.

REFERENCES

1. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol.* 12, 88-118
2. Frieden, C., and Colman, R. F. (1967) *J. Biol. Chem.* 242, 1705-1715
3. Cassman, M., and King, R. C. (1972) *Biochemistry* 11, 4937-4944
4. Ackers, G. K., and Halvorson, H. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 4312-4316
5. Mills, F. C., Johnson, M. L., and Ackers, G. K. (1976) *Biochemistry* 15, 5350-5362
6. Birkett, D. J., Dwek, R. A., Radda, G. K., Richards, R. E., and Salmon, A. G. (1971) *Eur. J. Biochem.* 20, 494-508
7. Yagi, K., Tanaka, F., Ohishi, N., and Morita, M. (1977) *Biochim. Biophys. Acta* 492, 112-125
8. Ingham, K., Saroff, H. A., and Edelhoch, H. (1975) *Biochemistry* 14, 4745-4750
9. Ingham, K., Saroff, H. A., and Edelhoch, H. (1975) *Biochemistry* 14, 4751-4758
10. Sasaki, K., Dockerill, S., Adamiak, D. A., Tickle, I. J., and Blundell, T. (1975) *Nature* 257, 751-757
11. Formisano, S., Johnson, M. L., and Edelhoch, H. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 3340-3344
12. Gratzer, W. B., and Beaven, G. H. (1969) *J. Biol. Chem.* 244, 6675-6679
13. Gratzer, W. B., Creeth, J. M., and Beaven, G. H. (1972) *Eur. J. Biochem.* 31, 505-509
14. Knott, G. D., and Reece, D. K. (1972) *Proceedings of the ONLINE '72 International Conference*, Vol. 1, pp. 497-526
15. Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math.* 11, 431-441
16. Koshland, D. E., Jr., Némethy, G., and Filmer, D. (1966) *Biochemistry* 5, 365-385
17. Nichol, L. W., Jackson, W. J. H., and Winzor, D. J. (1967) *Biochemistry* 6, 2449-2456
18. Frieden, C. (1971) *Annu. Rev. Biochem.* 40, 653-696
19. Ip, S. H. C., and Ackers, G. K. (1977) *J. Biol. Chem.* 252, 82-87