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Note

The estimation of glutaminyl deamidation and aspartyl cleavage rates in glucagon

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Abstract

The major hydrolytic degradation pathways of glucagon under acidic conditions are cleavage at Asp-9, Asp-15, and Asp-21, and deamidation at Gln-3, Gln-20, Gln-24, and Asn-28. The rate constants for these pathways were determined in the pH range 1–2.4 at 60 °C by kinetic data analysis of substrate and degradation product concentration–time profiles. Deamidation kinetics were determined using penta-peptide fragments of glucagon containing the labile amide residue. The accurate determination of the cleavage rate constants was confounded by the complexity of the degradation scheme of glucagon. Peptide cleavage kinetics were determined by degradation of glucagon and its cleavage fragments under identical conditions and the use of area-under-the-curve (AUC) and nonlinear regression methods of analysis. Glucagon degradation was first-order with respect to time and concentration in the range of 31–00 μM. Glutaminyl deamidation rate constants were first-order with respect to hydronium ion concentration and were similar for all three residues indicating a lack of sequence effects. The rate constants for Asp cleavage were not first-order with respect to hydronium ion concentration and cleavage at Asp-21 was slower than cleavage at Asp-9 and Asp-15 over the studied pH range.

Keywords: Deamidation; Aspartyl cleavage; Glucagon; Hydrolysis

Glucagon is a small protein composed of 29 amino acid residues ($HSQ_3GTFTSD_9YSKYLD_{15}SRRAQ_{20}D_{21}FVQ_{24}WLMN_{28}T$). In previous studies (Joshi et al., 2000), six major degradation pathways of glucagon were identified in acidic solutions; $n + 1$ peptide cleavage at Asp-9, Asp-15, and Asp-21, and deamidation at Gln-3, Gln-20, and Gln-24. Additionally, trace amounts of $n-1$ peptide cleavage at Asp-15 and deamidation at Asn-28 were detected. The objectives of the studies reported herein were to determine the order of glucagon degradation and to estimate the individual rate constants for each degradation pathway as a function of pH. The relationship between pH-dependent kinetics of individual pathways was used to make inferences regarding the mechanisms of glutaminyl deamidation and aspartyl cleavage.

Reaction order with respect to time was determined by degrading buffered solutions of glucagon (pH 1.0, 1.5, and 2.4) at 60 °C to approximately four half-lives. The linearity of the first-order plots at different pH demonstrated that the reaction was first-order with respect to time. Reaction order with respect to concentration was determined using the initial rate method (Connors, 1990). Glucagon solutions (31, 150, 300, and 581 μM) were prepared in 0.01 N HCl and degraded at 60 °C until 4–6% of the starting

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material was lost. Plots of initial degradation rate versus initial concentration yielded a straight line up to an initial concentration of 300 μM, which indicated that glucagon degradation was first-order with respect to concentration up to 300 μM. At higher concentrations, there was a negative deviation from the straight line, indicating a less than first-order reaction. This deviation from first-order behavior can be attributed to the stabilizing effect of glucagon self-association that is known to occur at higher concentrations (Beaven et al., 1969). The importance of these results was that first-order kinetic analyses were deemed appropriate for estimating individual rate constants. Also, studies conducted to measure the kinetics of glucagon loss were initiated with substrate concentrations in the range 28–31 μM at which first-order kinetics applied.

Degradation kinetics were studied by initiating reactions with glucagon, its cleavage fragments 10–9, 16–9, and 22–29 (resulting from $n + 1$ cleavage at Asp-9, Asp-15, and Asp-21, respectively), and the Gln containing peptides 1–5 and 17–21 at pH 1, 1.5 (0.13 and 0.04 M HCl), 2.0 and 2.4 (0.1 and 0.14 M phosphate). Initial substrate concentrations were approximately 30 μM; ionic strength was adjusted to 0.15 M with NaCl; and the degradation temperature was 60 °C. Reaction mixture samples were removed at pre-determined time intervals and frozen at −32 °C until they were analyzed by HPLC. All reaction mixtures were analyzed using either a Lichrospher RP-18, 5μ, 4.6 mm × 250 mm column or a Vydac C-4, 5μ, 4.6 mm × 250 mm column. Separation was achieved using gradients of 0.03% aqueous TFA with either acetonitrile or methanol. The analytical wavelength was 214 nm, column temperature was 35 °C, and the flow rate was 1 ml/min.

The deamidation rate constants were estimated from the Gln containing peptides 1–5, 17–21, and 22–29. Initial estimates were obtained using the method of initial rates (Connors, 1990). The initial estimates were then used in a nonlinear regression program (WinNonlin, Version 3.0) to accurately estimate the individual deamidation rate constants in the presence of parallel pathways of substrate loss. A typical fit for the deamidation reaction is shown in Fig. 1 and the rate constants and pH rate profiles are shown in Table 1 and Fig. 3a, respectively.

The estimation of the cleavage rate constants in glucagon was a significant experimental design and

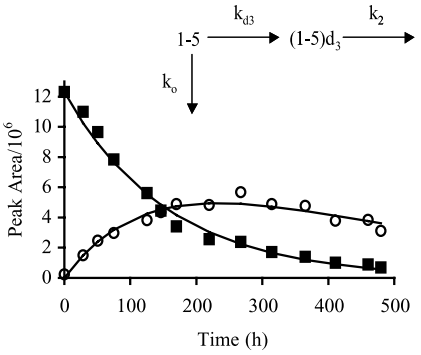


Fig. 1. Loss of 1–5 (■) and formation of its deamidation product (1–5)d_3 (○). Data points were experimentally determined and curves were generated by nonlinear regression using the scheme indicated.

data analysis problem. The observed rate constant for glucagon loss (k_{obs}) is the sum of the rate constants for loss by all parallel pathways including both cleavage and deamidation (Scheme 1). The complexity of the degradation scheme (especially the presence of multiple interconnecting pathways for generating cleavage products) necessitated a two-step approach for obtaining accurate rate constant estimates.

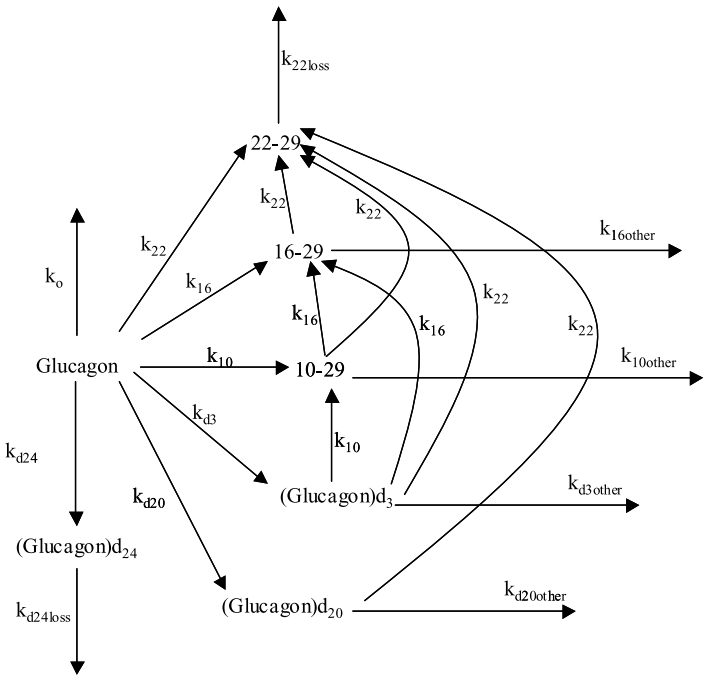
The two-step approach entailed:

(a) Preliminary estimation of rate constants using an area-under-the-curve (AUC) method of analysis (Notari and DeYoung, 1975).
(b) Fitting the complete model for glucagon degradation using nonlinear least squares regression and the initial estimates obtained in (a).

The AUC method of analysis can be used in a degradation scheme involving multiple parallel pathways of substrate loss to determine the fraction of starting material that is lost through a particular pathway. Con-

Table 1
Deamidation and cleavage rate constants for glucagon determined by nonlinear regression

pH	Deamidation rate constant ($\times 10^2$ h^{-1})				Cleavage rate constant ($\times 10^2$ h^{-1})		
	k_{d3}	k_{d20}	k_{d24}	k_{d28}	k_{10}	k_{16}	k_{22}
1.0	6.89	5.83	6.78	3.32	2.30	3.00	0.68
1.5	2.12	1.60	1.79	0.65	1.01	1.19	0.40
2.0	–	–	0.68	0.26	–	–	–
2.4	0.49	0.38	0.26	0.11	1.22	1.17	0.41



Scheme 1. The full model of glucagon degradation.

sider the reaction scheme

$$\underset{\downarrow k_0}{\mathrm{A}} \xrightarrow{k_1} \mathrm{B} \xrightarrow{k_2} \mathrm{C}$$

The fraction of A degrading to B ($f_{B/A}$) can be determined experimentally by: (a) starting a reaction with A (initial concentration $= A_0$) and measuring the AUC of B as it forms from A ($AUC_{B/A}$), (b) starting a reaction with B (initial concentration $= B_0$) with the same initial concentration as A in step (a) and measuring the AUC of its first-order loss (AUC_{Bloss}), (c) determining the fraction $f_{B/A}$ from the ratio of the two AUCs, i.e.

$$f_{B/A} = \frac{AUC_{B/A}}{AUC_{Bloss}} \tag{1}$$

In the more complex glucagon degradation scheme (Scheme 1), k_{10}, k_{16}, and k_{22} represent rate constants that describe the cleavage of the intact peptide at residues 9, 15 and 21 leading to the formation of peptide fragments 10–29, 16–29 and 22–29. Eq. (1) can be used to obtain the fraction of glucagon that degrades to 10–29 ($f_{10/G}$), 16–29 ($f_{16/G}$), and 22–29 ($f_{22/G}$). However, the cleavage products also arise from each other and from deamidation products. Therefore, the AUC obtained experimentally by integrating concentration–time profiles for each fragment represents a portion that forms directly from glucagon and a portion that forms circuitously via cleavage and deamidation products. Hence, in order to get an estimate of the cleavage rate constants k_{10}, k_{16}, and k_{22}, the portion of the AUC that is contributed by the cleavage and deamidation products needs to be subtracted from the experimentally determined AUC. This is described in Eqs. (2)–(4) which were used to obtain initial estimates for k_{10}, k_{16}, and k_{22}, respectively

$$\frac{k_{10}}{k_{obs}} = f_{10/G} - \left[\frac{k_{d3}}{k_{obs}}\frac{k_{10}}{k_{obs}-k_{d3}}\right] \tag{2}$$

$$\frac{k_{16}}{k_{obs}} = f_{16/G} - [f_{10/G} \times f_{16/10}] - \left[\frac{k_{d3}}{k_{obs}}\frac{k_{16}}{k_{obs}-k_{d3}}\right] \tag{3}$$

$$\frac{k_{22}}{k_{obs}} = f_{22/G} - [f_{16/G} \times f_{22/16}] - [f_{10/G} \times f_{22/10}] - \left[\frac{k_{d3}}{k_{obs}}\frac{k_{22}}{k_{obs}-k_{d3}}\right] - \left[\frac{k_{d20}}{k_{obs}}\frac{k_{22}}{k_{obs}-k_{d20}}\right] \tag{4}$$

The derivations for Eqs. (2)–(4) are provided in Appendix A.

The experimental design for estimating the cleavage rate constants was as follows. At each pH, four reactions were initiated with glucagon and its three cleavage fragments:

- Reaction 1: Loss of glucagon and formation of 10–29, 16–29, and 22–29.
- Reaction 2: Loss of 10–29 and formation of 16–29 and 22–29.
- Reaction 3: Loss of 16–29 and formation of 22–29.
- Reaction 4: Loss of 22–29.

These reactions gave rise to 10 concentration–time profiles that were used to obtain the fractions $f_{10/G}$, $f_{16/G}$, $f_{22/G}$, $f_{22/16}$, $f_{22/10}$, and $f_{16/10}$ using Eq. (1). The rate constants k_{obs}, k_{d3}, and k_{d20} were obtained from the first-order loss profiles of glucagon, 1–5, and 17–21, respectively. The initial estimates for k_{10}, k_{16}, and k_{22} were then calculated from Eqs. (2)–(4) and used in WinNonlin to estimate accurate cleavage rate constants by simultaneously fitting the concentration–time profiles for glucagon loss and degradation product appearance and loss using the model depicted in Scheme 1. The observed and model predicted concentration–time profiles obtained by nonlinear regression are shown in Fig. 2. The rate constants and pH rate profiles for $n + 1$ Asp cleavage are shown in Table 1 and Fig. 3b, respectively.

Differences in reaction rates at chemically identical amino acid residues can be attributed to either (a) a sequence effect in which the neighboring groups immediately surrounding the labile amino acid affect reaction rates, or (b) higher order structure effects in which the folding of the molecule can either enhance (Wright, 1991) or retard (Kossiakoff, 1988) reaction rates. Differences in the magnitude of rate constants for similar pathways can provide insight into whether primary sequence or conformation is the major determinant of reactivity.

The three Gln residues in glucagon (Ser-Gln_3-Gly, Ala-Gln_{20}-Asp, Val-Gln_{24}-Trp) have neighboring groups of varying nature, yet they reacted at similar rates (Fig. 3a). This lack of sequence effects is consistent with a direct hydrolysis mechanism (Patel and Borchardt, 1990). The rates of $n + 1$ cleavage were similar at Ser-Asp_9-Tyr and Leu-Asp_{15}-Ser but apparently lower at Gln-Asp_{21}-Phe (Fig. 3b). The lower propensity of Asp-21 to cleave may not be attributed to secondary structure effects because glucagon is predominantly random coil in dilute aqueous solutions. This has been shown through numerous studies using circular dichroism (Srere and Brooks, 1969), ^{1}H NMR (Boesch et al., 1978; Yi et al., 1992), and optical rotatory dispersion (Gratzer et al., 1968). Therefore, we believe the differences in $n + 1$ cleavage rates at the three Asp residues may be attributable to a primary sequence effect. The presence of hydroxyl groups near Asp-9 and Asp-15 (Ser-8, Tyr-10, and Ser-16) may be responsible for their higher reactivities because hydroxyl groups can act as potential hydrogen bonding partners for the Asp side-chain. The resulting effect on side-chain ionization may have an impact on reactivity. This hypothesis is further strengthened by the fact that the Asp-28 residue (resulting from deamidation of Asn-28) is next to a hydroxyl contain-

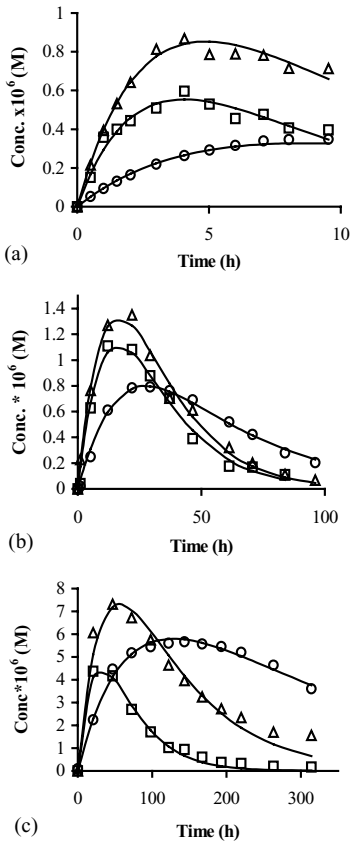


Fig. 2. Formation of 10–29 (□), 16–29 (△), and 22–29 (○) from glucagon at pH (a) 1.0, (b) 1.5, and (c) 2.4. Data points were experimentally determined and curves were generated by nonlinear regression using Scheme 1.

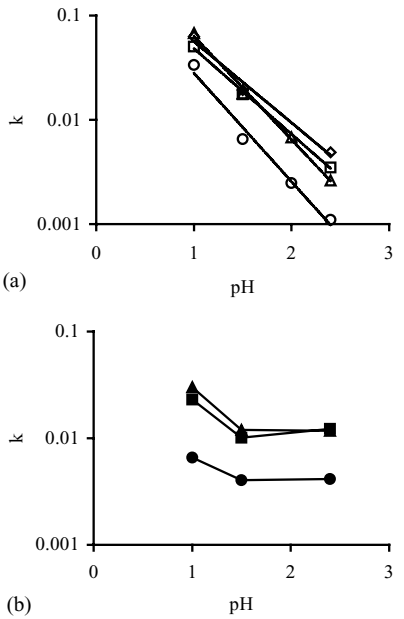


Fig. 3. pH rate profiles for (a) deamidation at Gln-3 (◇), Gln-20 (□), Gln-24 (△), and Asn-28 (○). (b) $n+1$ cleavage at Asp-9 (■), Asp-15 (▲), and Asp-21 (●).

ing residue (Thr-29) and is cleaved at rates similar to Asp-9 and Asp-15 (Joshi and Kirsch, 2002). Future studies will be aimed at testing this hypothesis.

Appendix A

Note: The following abbreviations were used in the equations that follow: "glucagon" has been abbreviated to "G", "10–29" has been abbreviated to "10", "16–29" has been abbreviated to "16", "22–29" has been abbreviated to "22".

In Scheme 1, the rate of change of concentration of 10–29 is given by

$$\frac{\mathrm{d}[10]}{\mathrm{d}t} = k_{10}\times[G] + k_{10}\times[G_{\mathrm{d3}}] - k_{10\mathrm{loss}}\times[10] \qquad (A.1)$$

where $k_{10\mathrm{loss}} = k_{16} + k_{22} + k_{10\mathrm{other}}$.

Taking the Laplace of Eq. (A.1) yields:

$$\overline{10} = \frac{k_{10}\bar{G}}{s + k_{10\mathrm{loss}}} + \frac{k_{10}\bar{G}_{\mathrm{d3}}}{s + k_{10\mathrm{loss}}} \qquad (A.2)$$

The expressions for $\overline{G}$ and $\bar{G}_{\mathrm{d3}}$ can be obtained by considering the rate of change of concentration of these species and taking the Laplace of the resulting differential equations. We get

$$\bar{G} = \frac{G_0}{s + k_{\mathrm{obs}}} \qquad (A.3)$$

$$\bar{G}_{\mathrm{d3}} = \frac{k_{\mathrm{d3}}G_0}{(s + k_{\mathrm{obs}})(s + k_{\mathrm{d3loss}})} \qquad (A.4)$$

where G_0 is the initial concentration of glucagon, k_{obs} the observed rate constant for loss of glucagon, k_{d3} the rate constant for deamidation at Gln-3, and k_{d3loss} the rate constant for loss of (glucagon)d_3.

Substituting Eqs. (A.3) and (A.4) into Eq. (A.2) and taking the inverse Laplace yields

$$\begin{aligned}[10] = {} & \frac{k_{10}G_0}{k_{10\mathrm{loss}} - k_{\mathrm{obs}}} \times (\mathrm{e}^{-k_{\mathrm{obs}}t} - \mathrm{e}^{-k_{10\mathrm{loss}}t}) \\ & + \frac{k_{10}k_{\mathrm{d3}}G_0}{(k_{\mathrm{d3loss}} - k_{\mathrm{obs}})(k_{10\mathrm{loss}} - k_{\mathrm{obs}})} \times (\mathrm{e}^{-k_{\mathrm{obs}}t}) \\ & + \frac{k_{10}k_{\mathrm{d3}}G_0}{(k_{\mathrm{obs}} - k_{\mathrm{d3loss}})(k_{10\mathrm{loss}} - k_{\mathrm{d3loss}})} \times (\mathrm{e}^{-k_{\mathrm{d3loss}}t}) \\ & + \frac{k_{10}k_{\mathrm{d3}}G_0}{(k_{\mathrm{obs}} - k_{10\mathrm{loss}})(k_{\mathrm{d3loss}} - k_{10\mathrm{loss}})} \times (\mathrm{e}^{-k_{10\mathrm{loss}}t}) \end{aligned} \qquad (A.5)$$

Integrating Eq. (A.5) from 0 to $\propto$ yields the experimentally observed AUC of 10–29 when the reaction is initiated with glucagon

$$\mathrm{AUC}_{10/\mathrm{G}} = \frac{k_{10}G_0}{k_{\mathrm{obs}}k_{10\mathrm{loss}}} + \frac{k_{\mathrm{d3}}k_{10}G_0}{k_{\mathrm{obs}}k_{\mathrm{d3loss}}k_{10\mathrm{loss}}} \qquad (A.6)$$

Dividing Eq. (A.6) by $\mathrm{AUC}_{10\mathrm{loss}} = G_0/k_{10\mathrm{loss}}$, we get

$$\frac{\mathrm{AUC}_{10/\mathrm{G}}}{\mathrm{AUC}_{10\mathrm{loss}}} = \frac{k_{10}}{k_{\mathrm{obs}}} + \frac{k_{\mathrm{d3}}k_{10}}{k_{\mathrm{obs}}k_{\mathrm{d3loss}}}$$

Since glucagon and $(\mathrm{glucagon})_{\mathrm{d3}}$ are only different in their third residue, they will degrade by similar pathways. Hence, we can write $k_{\mathrm{d3loss}} = k_{\mathrm{obs}} - k_{\mathrm{d3}}$

$$f_{10/\mathrm{G}} = \frac{k_{10}}{k_{\mathrm{obs}}} + \left(\frac{k_{\mathrm{d3}}}{k_{\mathrm{obs}}}\frac{k_{10}}{k_{\mathrm{obs}} - k_{\mathrm{d3}}}\right) \qquad (A.7)$$

Rearranging Eq. (A.7), we get Eq. (2), where k_{10} is the rate constant for $n+1$ cleavage at Asp-9, k_{obs} the observed rate constant for glucagon loss, k_{d3} the rate constant for deamidation at Gln-3, and $f_{10/\mathrm{G}}$ the fraction of glucagon that degrades to 10–29. Eq. (2) can be used to obtain an initial estimate of k_{10} since k_{obs} and k_{d3} are known and $f_{10/\mathrm{G}}$ can be experimentally obtained using Eq. (1). It can be seen from

Eq. (2) that the accurate estimation of k_{10} was obtained by experimentally determining the total AUC of 10–29 originating from glucagon ($f_{10/G}$) and subtracting from it the effect of the pathway glucagon → (glucagon)d3 (represented by k_{d3}/k_{obs}), and the effect of the pathway (glucagon)d3 → 10–29 (represented by $k_{10}/(k_{obs} - k_{d3})$).

In a similar manner, we can obtain the expression for the experimentally observed AUC of 16–29 when the reaction is initiated with glucagon

$$\mathrm{AUC}_{16/G} = \frac{k_{16}G_0}{k_{obs}k_{16loss}} + \frac{k_{10}k_{16}G_0}{k_{obs}k_{10loss}k_{16loss}} + \frac{k_{d3}k_{16}G_0}{k_{obs}k_{d3loss}k_{16loss}} \quad (A.8)$$

Dividing Eq. (A.8) by $\mathrm{AUC}_{16loss} = G_0/k_{16loss}$, we get

$$\frac{\mathrm{AUC}_{16/G}}{\mathrm{AUC}_{16loss}} = \frac{k_{16}}{k_{obs}} + \frac{k_{10}k_{16}}{k_{obs}k_{10loss}} + \frac{k_{d3}k_{16}}{k_{obs}k_{d3loss}} \quad (A.9)$$

Since glucagon and $(\text{glucagon})_{d3}$ are only different in their third residue, they will degrade by similar pathways. Hence, we can write $k_{d3loss} = k_{obs} - k_{d3}$. Also, we can write $k_{10}/k_{obs} = f_{10/G}$, and $k_{16}/k_{10loss} = f_{16/10}$. Rearranging Eq. (A.9), we get Eq. (3), where k_{16} is the rate constant for $n + 1$ cleavage at Asp-15, $f_{16/G}$ the fraction of glucagon that degrades to 16–29, and $f_{16/10}$ the fraction of 10–29 that degrades to 16–29. Eq. (3) can be used to obtain an initial estimate for k_{16} since k_{obs} and k_{d3} are known, and $f_{16/G}$, $f_{10/G}$, and $f_{16/10}$ can be experimentally determined using Eq. (1). It can be seen from Eq. (3) that the accurate estimation of k_{16} was obtained by experimentally determining the total AUC of 16–29 originating from glucagon ($f_{16/G}$) and subtracting from it the effect of the pathway 10–29 → 16–29 (represented by $f_{10/G} \times f_{16/10}$), and the effect of the pathways glucagon → (glucagon)d3 (represented by k_{d3}/k_{obs}) and (glucagon)d3 → 10–29 (represented by $k_{16}/(k_{obs} - k_{d3})$).

Similarly, we can also obtain an expression for the experimentally observed AUC of 22–29 when the reaction is initiated with glucagon

$$\mathrm{AUC}_{22/G} = \frac{k_{22}G_0}{k_{obs}k_{22loss}} + \frac{k_{16}k_{22}G_0}{k_{obs}k_{16loss}k_{22loss}} + \frac{k_{10}k_{22}G_0}{k_{obs}k_{10loss}k_{22loss}} + \frac{k_{d3}k_{22}G_0}{k_{obs}k_{d3loss}k_{22loss}} + \frac{k_{d20}k_{22}G_0}{k_{obs}k_{d20loss}k_{22loss}} \quad (A.10)$$

Dividing Eq. (A.10) by $\mathrm{AUC}_{22loss} = G_0/k_{22loss}$, we get

$$\frac{\mathrm{AUC}_{22/G}}{\mathrm{AUC}_{22loss}} = \frac{k_{22}}{k_{obs}} + \frac{k_{16}k_{22}}{k_{obs}k_{16loss}} + \frac{k_{10}k_{22}}{k_{obs}k_{10loss}} + \frac{k_{d3}k_{22}}{k_{obs}k_{d3loss}} + \frac{k_{d20}k_{22}}{k_{obs}k_{d20loss}} \quad (A.11)$$

Since glucagon, $(\text{glucagon})_{d3}$ and $(\text{glucagon})_{d20}$ are only different in their third residue, they will degrade by similar pathways. Hence, we can write $k_{d3loss} = k_{obs} - k_{d3}$ and $k_{d20loss} = k_{obs} - k_{d20}$. Also, we can write $k_{16}/k_{obs} = f_{16/G}$, $k_{22}/k_{16loss} = f_{22/16}$, $k_{10}/k_{obs} = f_{10/G}$, and $k_{22}/k_{10loss} = f_{22/10}$. Rearranging Eq. (A.11), we get Eq. (4), where k_{22} is the rate constant for $n + 1$ cleavage at Asp-21, k_{d20} the rate constant for deamidation at Gln-20, $f_{22/G}$ the fraction of glucagon that degrades to 22–29, $f_{22/16}$ the fraction of 16–29 that degrades to 22–29, and $f_{22/10}$ the fraction of 10–29 that degrades to 22–29. Eq. (4) can be used to obtain an initial estimate for k_{22} since k_{obs}, k_{d3}, and k_{d20} are known, and $f_{22/G}$, $f_{16/G}$, $f_{10/G}$, $f_{22/16}$, and $f_{22/10}$ can be experimentally determined using Eq. (1). It can be seen from Eq. (4) that the accurate estimation of k_{22} was obtained by experimentally determining the total AUC of 22–29 originating from glucagon ($f_{22/G}$) and subtracting from it the effect of the pathways 16–29 → 22–29 (represented by $f_{16/G} \times f_{22/16}$), 10–29 → 22–29 (represented by $f_{10/G} \times f_{22/10}$), glucagon → (glucagon)d3 (represented by k_{d3}/k_{obs}) and (glucagon)d3 → 22–29 (represented by $k_{22}/(k_{obs} - k_{d3})$), and glucagon → (glucagon)d20 (represented by k_{d20}/k_{obs}) and (glucagon)d20 → 22–29 (represented by $k_{22}/(k_{obs} - k_{d20})$).

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